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STUDIES ON HYPOALBUMINEMIA PRODUCED BY PROTEIN-DEFICIENT DIETS

III The Correction of Hypoalbuminemia in Dogs by Means of Large Plasma Transfusions

By ROBERT ELMAN M.D and HARRIET WOLF DAVEY

(*From the Department of Surgery, Washington University School of Medicine, St Louis*)

(Received for publication, August 28, 1942)

Plasma transfusions are often used clinically to correct hypoproteinemia resulting from malnutrition although the results are often disappointing in the severe cases It seemed advisable, therefore, to study experimentally the effect of intravenous plasma in dogs depleted by a non protein diet which has been shown by Weech (1) and ourselves (2) to produce a rapid fall in the albumin fraction of the blood Holman, Mahoney, and Whipple (3) were the first to study the metabolic effect of plasma transfusions in the dog and they showed that protein given in this way led to positive nitrogen balance but only when dog plasma was used These findings were confirmed by later studies (4, 5) More recently Kremen *et al* have reported similar findings with human plasma in human beings but not with bovine plasma (6) In protein-depleted dogs Shearburn (7) found that one plasma transfusion calculated to restore the plasma protein deficiency had only a transient influence on plasma volume and no effect on the hypoproteinemia, whereas smaller amounts given once or twice a day for 2 weeks led to a gradual return of the plasma protein to normal

Procedures

The dietary method used to produce hypoalbuminemia was similar to that devised by Weech (1) except that a solution was used and administered by gavage twice daily (2) The energy value was 50 calories per kilo per day and the nitrogen intake due largely to vitamin B complex was under 20 mg per kilo per day A period of 3 weeks was used for depletion the 4th week was used for therapy and the depletion then continued for 2 more weeks Thus each experiment lasted 6 weeks. Under such a non protein régime hypoalbuminemia rapidly develops except that occasionally it is masked by hemoconcentration which is revealed by a rise in the hematocrit reading (8) However, in the present experiments plasma volumes were measured to circumvent this state of affairs, the method used was described in a previous paper in this series (9)

The amount of plasma given during the 4th week was determined by measuring its nitrogen content. A dose of 0 5 gm of nitrogen per kilo per day was given, which was the same we used in other regeneration experiments in which hydrolyzed protein was used (2) In terms of protein this represented a little over 3 gm per kilo per day, which is somewhat greater than 2.5 gm employed by Weech. It was

necessary to give about 500 to 600 cc of dog plasma per day, the rate of injection was about 100 cc. per hour No protein appeared in the urine The plasma was obtained from donor dogs by centrifuging their citrated blood after bleeding The methods for determining the hematocrit reading, and the albumin and globulin,

TABLE I

Blood Changes and Nitrogen Balance in Dietary Hypoalbuminemia As Influenced by Large Plasma Transfusions

Each dog received during the week (4) of therapy plasma amounting to exactly 0 5 gm N/kg /day or roughly 600 cc per day N intake due entirely to vitamins and plasma

Blood changes						Nitrogen balance					
Day	Hematocrit reading	Albumin	Globulin	P V	T C.A	Week	Intake	Urinary output	Balance	Urine output	Remarks
	per cent	*gm per cent*	*gm per cent*	*cc*	*gm*		*gm*	*gm*	*gm*	*cc*	
1	45 7	3 75	3 44	—	—	0	—	—	—	—	Dog C3
7	—	—	—	—	—	1	1 26	—	—	—	9 6 kg
14	44 3	3 23	4 06	—	—	2	1 26	—	—	—	
21	45 4	2 93	4 20	482	14 1	3	1 26	11 11	−9 85	4030	
28	39 8	4 73	4 32	616	29 1	4	35 69	12 32	+23 37	8530	
35	36 1	4 08	3 90	—	—	5	1 26	24 58	−23 32	4460	
42	39 6	3 29	3 05	379	12 5	6	1 26	—	—	—	
1	49 8	3 56	2 05	—	—	0	—	—	—	—	Dog C4
7	—	—	—	—	—	1	1 33	—	—	—	9 8 kg
14	47 1	3 08	2 26	—	—	2	1 33	—	—	—	
21	50 0	2 79	2 97	515	14 4	3	1 33	12 73	−11 40	4170	
28	33 0	4 32	3 61	746	32 2	4	36 35	15 88	+20 47	9480	
35	31 4	3 84	2 62	—	—	5	1 33	25 62	−24 29	4420	
42	36 5	3 16	2 42	515	16 3	6	1 33	—	—	—	
1	46 4	3 54	2 12	—	—	0	—	—	—	—	Dog D1
7	—	—	—	—	—	1	1 47	—	—	—	10 8 kg
14	52 3	3 01	2 37	—	—	2	1 47	—	—	—	
21	53 1	3 21	2 20	442	14 2	3	1 47	19 54	−18 07	3890	
28	37 3	4 10	3 97	690	28 3	4	39 27	16 43	+22 84	6610	
35	44 0	3 89	3 22	497	19 3	5	1 47	34 35	−32 88	5760	
42	—	—	—	—	—	6	1 47	27 40	−25 93	5180	

P V—plasma volume, T C.A—total circulating albumin

have been described previously (2) During the periods of urine collection the dogs were kept in metabolism cages and the usual precautions taken

FINDINGS

The really large plasma transfusions were tolerated without event No symptoms or signs were noted in the dogs, aside from a pronounced diuresis

Thus "toxic" symptoms as observed in other reported experiments (5) did not occur The data on the blood changes and nitrogen balance are listed in Table I The increase in the plasma proteins after the week of plasma was striking and affected both albumin and globulin, mostly the former There was also a striking increase in plasma volume so that the total circulating albumin doubled in all experiments Urinary nitrogen remained uninfluenced during the week of plasma injections, indicating a complete retention and the achievement of a remarkable positive nitrogen balance. However, during the weeks following the injections the output of urinary nitrogen increased and in one experiment was so great as to wipe out all of the nitrogen retained during the week of plasma injections In the course of 2 weeks the plasma volume returned to its previous level as did the albumin and globulin concentrations. The diuresis which occurred during the week of plasma transfusions doubled the urinary secretion

COMMENT

The findings in these experiments were striking and in some respects unexpected That the plasma proteins and volumes should increase might have been foreseen but the magnitude of the changes was surprising Doubtless the diuresis was a manifestation of the increased colloidal osmotic pressure produced by the large increases in the concentration of plasma proteins Although nutritionally the introduced amount of plasma as nitrogen was not excessive, in terms of plasma transfusions it was tremendous The protein intake was 3 gm per kilo per day, which is a large but not an unusual amount from the nutritional point of view, yet it represents a volume of plasma greater than the plasma volume already present. In other words, the daily injection doubled the amount of normally circulating plasma This contrast is of some interest when one considers plasma as a means of supplying protein nourishment parenterally

Totally unexpected was the large excretion of urinary nitrogen following the week of plasma injections One cannot escape the inference that all injected plasma,—at least in the large doses used in these experiments, was *not* utilized as nitrogenous nourishment for the rest of the body By comparing the amount of plasma injected with the increase of circulating protein observed, it is obvious that during the week of injections but a small part of the injected plasma protein remained in the blood The actual percentages are, in the three experiments, 9 9, 13.5, and 13.5 per cent (Table II) That the rest was taken up by the tissues seems obvious for there was no increase in urinary nitrogen during this period It is conceivable, of course, that the protein was slowly catabolized in these tissues, the end products being stored for a while but eventually excreted in the urine. Nevertheless, the present data cast doubt on the assumption that the body is able to utilize plasma pro-

teins in the building of protein tissue elsewhere, at least when injected in large amounts In contrast to these findings with plasma, previous experiments showed no increased nitrogen output in the week following the injection of the same amount of nitrogen as hydrolyzed casein It is of interest to mention the findings of Schoenheimer *et al* (10), who found in the rat that plasma proteins are being continuously destroyed and that their half life is about 2 weeks

The present findings are not in agreement with the observations of Holman, Mahoney, and Whipple (3), who found no excessive nitrogen excretion during 5 days following a 2 week period of plasma transfusions However, the daily amount of plasma they injected was only about one-third that used in the present experiments If the difference is actually due to this circumstance, it suggests a limitation of the amount of plasma introduced intravenously which can be utilized each day as protein alimentation The correction of nutritionally induced hypoalbuminemia with small repeated plasma injections

TABLE II

Proportion of Injected Protein Remaining in the Blood after the Week of Plasma Transfusions

Dog No	Before plasma transfusions			Plasma protein injected	After plasma transfusions			Injected plasma protein remaining in plasma
	P V	T P	T C P		P V	T P	T C P	
	cc	*gm per cent*	*gm*	*gm*	*cc*	*gm per cent*	*gm*	*per cent*
C3	482	7 13	34 4	215	616	9 05	55 7	9 9
C4	515	5 76	29 7	219	746	7 93	59 2	13 5
D1	442	5 41	23 9	236	690	8 07	55 7	13 5

P V—plasma volume, T P—total protein concentration, T C P—total circulating protein

already noted (7) is of interest in this connection In view of the use of plasma transfusions to combat hypoproteinemia of nutritional origin in the human being, further observations seem indicated

CONCLUSIONS

1 It proved possible to correct dietary hypoalbuminemia in dogs by large plasma transfusions (about 50 cc per kilo per day) After 1 week of injections the increase of plasma protein exceeded the normal level although but 10 to 13 per cent of the injected protein remained in the blood There was an associated increase in plasma volume and a marked diuresis During the following 2 weeks the plasma volume and protein returned to their previous low levels

2 The nitrogen introduced was retained and produced no change in urinary excretion during the week of plasma injections, but in the following 2 weeks there was an increased nitrogen output The inference would seem to be that the large amounts of plasma injected in these experiments were not perma-

nently utilized by the body as nitrogenous nourishment but after some delay were largely excreted in the urine.

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SYNERGISTIC ACTION OF HEMOPHILUS INFLUENZAE SUIS AND THE SWINE INFLUENZA VIRUS ON THE CHICK EMBRYO

By FREDERIK B BANG M.D

(*From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey*)

PLATES 1 AND 2

(Received for publication, August 20, 1942)

In 1931 Shope (1–3) established that swine influenza was caused by the concerted action of a virus and a bacterium. The clinical and pathological similarities of swine and human influenzas have led several workers (4–6) to suppose that human pandemic influenza might also be caused by two agents acting in concert. But no synergism has been effected in the laboratory animals commonly susceptible to the human influenza virus (7), nor has the inoculation of ferrets and mice simultaneously with *Hemophilus influenzae suis* and swine virus resulted in a more severe disease than that produced by virus alone (8, 9)

Elkeles (10) found pigs to be susceptible to human influenza A virus and showed that the addition of cultures of *H influenzae* either human or swine, produced a more severe disease. Shope and Francis (11) corroborated this for the swine *Hemophilus* but found that "the increased severity of the pneumonia produced by the swine virus and bacterium [compared to that of human virus and swine bacterium] seems to constitute a significant difference between the strains of human and swine influenza virus studied." They did not test human strains of *H influenzae* Other workers (12) have found that human *H influenzae* has no enhancing effect on the filtrate disease of swine, whether produced by swine or human virus but they admit that this may be due to the fact that their strains of human *Hemophilus* fail to persist in the pig

The lack of evidence for a bacterial component in interpandemic influenza does not preclude the possibility that the pandemics are due to two agents acting in concert for the two types of disease differ greatly in severity A bacterial component in pandemic influenza is indicated by the frequency with which *H influenzae* was obtained in certain Army camps during the epidemic of 1918 Thus the whole question remains open and perhaps will only be settled during the next pandemic.

The work just summed up shows that the failure to obtain a demonstrable synergism for human influenza virus in experimental animals is paralleled by an inability to transpose the complex swine infection to other animals It seems possible that if the latter disease can be reproduced in a different host this host may prove to be a suitable test animal for tests of synergism in human influenza. The chick embryo is susceptible to infection with a number of species of *Hemophilus*, and the pathological response in all cases mimics the natural disease pattern (13–15) Burnet (16) has demonstrated that the

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human influenza virus is pneumotropic in the chick embryo, for it will destroy most of the bronchial alveolar epithelium following intra-amniotic injection

We have found that the combined infection of embryos with swine influenza virus and *H influenzae suis* produces a highly lethal infection, while neither one alone kills many embryos Infection with the virus allows the *Hemophilus* to persist longer than it does in normal embryos Finally the combined infection has a selective destructive effect on the embryo lungs

Materials and Methods

All strains of *Hemophilus influenzae suis* and swine influenza virus used in these experiments were kindly furnished by Dr Shope

H influenzae suis strain F was isolated from swine lungworms in 1941 It has been consistently capable of producing swine influenza when combined with the virus It was used after 30 transfers on blood agar

H influenzae suis strain 451 was isolated in 1928 by Lewis and Shope (2) It has been carried on artificial media since then with loss of virulence (17)

The *Hemophilus* was cultured and transferred as routine in 1 cc of defibrinated horse blood at the base of a plain agar slant

The swine influenza virus, V 15, was isolated in 1930 and passed through 176 mouse passages

The PR 8 strain of human influenza virus (influenza A) was isolated from material from Puerto Rico by Francis (18) and is mouse-adapted

Influenza B (Lee strain) was isolated by Francis (19)

Cultures of *Hemophilus* were killed by heating to 55–60°C for 30 minutes (20)

Various ages of embryos and methods of inoculation were tested in preliminary trials before 9 day embryos were selected Some of these tests are shown in Table I The 9 day embryo has the advantage that the virus infection can persist for a number of days before the embryo gains the ability to regulate its own temperature and before it tends to become naturally resistant to many bacterial and virus infections Embryos 9 days old were opened by cutting a window in the side of the egg and allowing the exposed chorioallantoic membrane to settle slowly (21) This was inoculated with a drop of the virus suspension, usually either in the form of fresh Berkefeld filtrates of diseased mouse lungs, or allantoic fluid from a previously infected embryo Occasionally the embryo was inoculated with a saline suspension of an infected chorioallantoic membrane Control embryos were similarly prepared but not inoculated with virus The window was covered with Scotch tape and the embryos were incubated for 24 hours, allowing the virus to gain a foothold before the *Hemophilus* was added One-half of the membranes infected with swine influenza virus were then inoculated with *H influenzae suis* and reincubated The control membranes were also inoculated with *Hemophilus* Each experiment thus contained at least three groups of embryos those receiving virus alone, those receiving virus and then *Hemophilus*, and those receiving *Hemophilus* alone When it was desired to test the relative effects of two viruses, five groups were set up, as may be seen in Table II The presence of *Hemophilus* on the membrane of embryos killed by the combination of bacterium and virus was always demonstrated by film, frequently in culture

Tests were also made in each experiment for the presence of virus. One method was inoculation of allantoic fluid from one or two of the embryos intranasally into at least two mice under light ether anesthesia. Another test was further embryo passage followed by intranasal mouse inoculation, the test being called positive if typical gross lesions developed in the lungs in 3 to 5 days. The agglutination of the chick's own red blood cells (22) was also useful in detecting the presence of virus, but was only used in conjunction with the above tests.

Later in the study embryos were fixed in Zenker's fixative plus 10 per cent acetic acid and sections stained with hematoxylin and eosin. Only live embryos were used

TABLE I

Lethal Effect of Hemophilus influenzae suis and Swine Influenza Virus within 48 Hours of Inoculation

Mode of inoculation	Age of embryo	Embryo passage of virus	Age of *Hemophilus* culture	Embryos inoculated with: Virus alone, No. of embryos: Dead	Alive	*Hemophilus* alone, No. of embryos: Dead	Alive	Virus + *Hemophilus*, No. of embryos: Dead	Alive
	days		*hrs*						
Membrane simultaneous	10	7	48	0	5	1	4	4	2
Amniotic and allantoic fluid	12	5	72*	0	7	2	4	5	3
Membrane, separate	9	2	48	0	7	0	6	6	1
" "	9	3	48	2	5	2	5	6	0
" "	9	4	24	1	2	2	4	4	0
	9	2	24	0	10	1	9	4	6
"	9	5	36	0	9	0	7	3	5
Totals for 9 day embryos				3	33	5	31	23	12
Mortality *per cent*				8.4		13.9		65.4	

* 72 hour amniotic fluid from infected embryo by amniotic route.

although in certain cases moribund embryos were fixed to demonstrate the maximum pathological changes. Heart blood cultures were taken from other embryos after immersing them for 1 minute in Zenker's fluid. The chest wall was opened, the heart seared, and punctured with a capillary pipette (23).

RESULTS

Table I shows that the combination of *Hemophilus* and virus consistently kills a greater proportion of embryos than does either one alone. The combined figures also show that the percentage of embryos killed by the combination is three times as great as the sum of those killed by the two agents inoculated separately, so that a synergistic effect is indicated. The mortality figures in Table I and subsequent tables cover a period of 48 hours after inoculation

with *Hemophilus*, but it is to be emphasized that many experiments were observed for several days thereafter and no significant increase in mortality was noticed in any of the series. Actually embryos in the combination series were usually dead within 24 hours after the addition of the *Hemophilus*.

The data in Table I also suggest that the mode of inoculation and the source of *Hemophilus* make little difference. In later experiments the *Hemophilus* cultures were arbitrarily added 1 day after inoculation with the virus.

Certain minor variations in mortality occur from experiment to experiment. Many of these may be due to variations in absorption of toxins from the bacillus, for it was later found that mortality increased if the *Hemophilus* blood suspension was first diluted in saline, so that a greater volume of fluid, con-

TABLE II

Effect of Early and Later Passage of Swine Virus on Mortality of Embryos

Early passage							Late passage				
No. of embryo passage	Virus alone		Virus + *Hemophilus*		*Hemophilus* alone		No. of embryo passage	Virus alone		Virus + *Hemophilus*	
	No. of embryos		No. of embryos		No. of embryos			No. of embryos		No. of embryos	
	Dead	Alive	Dead	Alive	Dead	Alive		Dead	Alive	Dead	Alive
2	0	7	3	5	0	6	9	0	7	5	3
3	1	5	5	1	1	4	11	2	5	6	1
Total	1	12	8	6	1	10		2	12	11	4
Mortality, *per cent*	7.7		57.1		9.1			14.3		73.3	

taining the same number of organisms, could be added. This diluted suspension covered a larger area of the membrane, presumably allowing for more absorption. A similar effect has been noted in some recent (unpublished) work on the growth of the gonococcus on the chorioallantoic membrane. An increase in mortality was also produced by adding 1 cc. of saline directly onto the drop of the *Hemophilus* blood culture.

The experiments in Table II were carried out to test the possibility that on serial embryo passage the virus of swine influenza lost the ability to act synergistically with *Hemophilus*. The difference in mortality between early and late passages is not statistically significant.

Human Influenza Virus

The synergistic effect in the embryo can only be considered related to the phenomenon in the pig if some degree of specificity is demonstrable. Table III summarizes a series of experiments comparing the effect of inoculating the

same suspension of *H influenzae suis* on groups of embryos previously infected with human and swine influenza virus Two representative human strains were studied Each horizontal line represents a separate experiment The far greater effect of the swine virus indicates that there is some specificity in the reaction in the embryo, just as there is in the pig

Recently Isolated and Stock Cultures of Hemophilus

Buddingh and Polk (23) found in work with the meningococcus in the embryo that avirulent stock cultures invaded poorly and killed few embryos, while recently isolated strains invaded tissue and produced septicemia and meningitis *H influenzae suis* is apparently rather slow to lose its ability to act in

TABLE III

A Comparison between Human and Swine Influenza Virus

Strain	Human virus: No of embryo passage	Human virus: Virus alone, No of embryos, Dead	Virus alone, Alive	Virus + *Hemophilus*, No of embryos, Dead	Virus + *Hemophilus*, Alive	*Hemophilus* alone, No of embryos, Dead	*Hemophilus* alone, Alive	Swine virus: No of embryo passage	Swine virus: Virus alone, No of embryos, Dead	Virus alone, Alive	Virus + *Hemophilus*, No of embryos, Dead	Virus + *Hemophilus*, Alive
PR 8	7	0	6	1	5	1	4	3	1	4	5	1
PR 8	2	0	8	4	5	0	8					
PR 8	1	1	7	0	8	2	8	7	2	9	7	4
Lee (B)	1	1	9	1	9	0	10	8	1	6	4	5
" "	3	0	8	1	8	1	8	8	5	4	9	0
Total		2	38	7	35	4	38		9	23	25	10
Mortality *per cent*				16 7							71 4	

concert with the virus in the pig Strain 451 produced typical swine influenza after more than 175 passages, although it was no longer able to produce influenza by contact (17) Later this strain (No 451) lost its ability to produce typical influenza when inoculated with the virus (24), and has since been carried on artificial media by Dr Shope for a total of more than 650 transfers for 14 years It was compared with the recently isolated swine strain which has consistently produced the complex disease (24) No great difference was found between the two strains when 24 hour cultures were inoculated on identically prepared swine influenza embryos (Table IV)

The establishment of the synergistic action of *Hemophilus* and influenza virus in the embryo is of interest because it allows a study and analysis of some of the factors concerned. From a study of the pathology of swine influenza Shope (6) has suggested the probability "that the activities of both the virus and the organism are influenced by the concomitant presence of the other

agent in the respiratory tract and that both actually contribute to the lesions of swine influenza"

TABLE IV

The Comparative Effect of Recently Isolated and Stock Strains of Hemophilus on Mortality of Embryos

	Recently isolated *Hemophilus*				Virus alone		Old stock *Hemophilus*			
	Alone		+ virus				Alone		+ virus	
	No of embryos		No of embryos		No of embryos		No of embryos		No of embryos	
	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive
	1	9	4	6	0	10	2	8	2	8
	0	7	3	5	0	9	0	7	3	4
	2	7	8	2	1	9	3	7	8	2
Total	3	23	15	13	1	28	5	22	13	14
Mortality, *per cent*			53						48	

TABLE V

*Persistence of H influenzae suis on Normal and Influenza Embryos**

Hemophilus on normal membranes		*Hemophilus* on membranes containing 4th passage swine influenza virus	
48 hrs.		48 hrs	
Appearance	Film	Appearance	Film
Slightly cloudy	–	Clear	–
" "	–	Ulcer 1 5 cm	++++
Clear, ulcer 0 5 cm	–	Slight ulcer	–
Slightly cloudy	–	Ulcer 1 5 cm	++
Clear	–	" 1 "	++++
Slight ulcer	–	" 2 "	++++
Clear	–	" 3 "	++++
"	–	" 2 "	–
		Dead	+++
		Small ulcer	+++

* 2 drops of an emulsion of *H influenzae suis* from 10 cc. of saline washings from a 30 hour chocolate agar slant were inoculated on normal and influenza embryos

Neither virus nor *Hemophilus* was demonstrable by the 5th day after inoculation

In the experiments outlined in Table I showing the increased mortality produced by the combination of agents, a large number of organisms was almost always used in a small volume of the inoculum (1 or 2 drops of undiluted blood from the standard culture) If fewer organisms are added, or if a dilute suspension of organisms from a chocolate agar slant is added to the membrane

infected with swine influenza virus, few or none of the embryos die The organisms, however, persist for several days longer than they do on normal embryos and produce larger ulcers with exudate Table V shows the results of one of the two experiments

It is evident from these results that infection of the chorioallantoic membrane with the virus of swine influenza predisposes to infection with *H influenzae suis*

Effect of Killed Hemophilus

Embryos inoculated with the combination of agents often die 14 to 16 hours after the addition of *Hemophilus* This suggests that death is due to products of bacterial growth rather than to invasion of the embryos, especially since the

TABLE VI

Effect of Killed Hemophilus on Mortality of Embryos

	Live *Hemophilus*				Virus alone		Dead *Hemophilus*			
	Hemophilus alone		*Hemophilus* + virus				*Hemophilus* alone		*Hemophilus* + virus	
	No of embryos		No of embryos		No of embryos		No of embryos		No of embryos	
	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive
			4	4	2	5	0	7	3	4
	1	10	5	5	1	10	1	9	9	2
Total	1	10	9	9	3	15	1	16	12*	6
Mortality *per cent*			50						66 6	

* Cultures of these embryos were negative for all bacteria including *Hemophilus*

size of the area covered by the inoculum affects the mortality Further, blood cultures are usually negative

To determine the effect of killed bacteria, 24 hour cultures of *H influenzae suis* were heated at 55–60°C in a sealed glass tube for ½ hour (20) Embryos infected with swine influenza virus, and normal controls, were inoculated with this emulsion. Control embryos received untreated 24 hour cultures Results of two groups of experiments are summarized in Table VI. These results show that some product of the bacterial metabolism may act synergistically with the virus

Pathology

Swine influenza is essentially a lobular pneumonia with a characteristic histopathology Shope (1) describes it as follows

"The small bronchi and terminal bronchioles were filled with a polymorphonuclear leucocytic exudate. Bacteria were never numerous in the exudates. There was

an extensive peribronchial round cell infiltration Alveoli were collapsed and frequently contained desquamated epithelial cells, small numbers of mononuclear wandering cells Leucocytes and red cells were not found regularly in the alveoli "

In pigs which have the filtrate disease,

"the bronchial epithelium was damaged, there was a heavy peribronchial cuffing with round cells and the alveolar walls were wrinkled, thickened, and infiltrated by round cells The collapsed alveoli were usually free of cells and, in contrast to swine influenza, no leucocytes are present, as rule, in the lumen of bronchi or in the alveoli of involved areas of lung" (1)

In the gross the chorioallantoic membranes of embryos infected with swine influenza virus show little unusual other than a slightly edematous thickening and a little whitish exudate on the surface No definite pocks are noticeable A few embryos die with extensive hemorrhage and thrombosis

Histologically the chorioallantoic membranes of 10 to 12 day embryos infected with the virus of swine influenza show several unusual changes Within the first 2 days a marked and rather extensive phagocytosis of the chick's own red blood cells occurs Phagocytic cells may be found containing up to 6 or 8 red blood cells each This occurs usually near a small hemorrhage in the absence of any noticeable inflammatory reaction (Fig 4) Since phagocytosis is often considered as a foreign body reaction, the obvious explanation is that the red blood cells have been coated with virus, as described by Hirst (22) in the agglutination phenomenon, and are thus foreign to the embryo However that may be, we have seen this same phagocytosis of red blood cells in 10 day embryos infected with equine encephalomyelitis but have not seen it in other embryo infections

By the 3rd day of infection with the swine virus the chorioallantoic membrane is considerably altered Besides the scattered destruction of the ectoderm with a moderate polymorphonuclear response, there are heavy ribbons of infiltrating cells in the mesoderm (Fig 5) Most of this appears related to blood vessels, either alongside or surrounding them The predominant cells are mononuclears tightly packed together, with polymorphonuclears dispersed among them Occasionally the latter occur separately as tight clumps

We have not noted the foci of ectodermal destruction described by Burnet (25) as pocks These changes, however, occur in embryo-adapted virus, and we here are dealing with recent embryo passages

Six days after infection, when virus is no longer demonstrable in the allantoic fluid of the embryo, the membrane may show more chronic changes The ectoderm is greatly thickened, with layers of cells heaped on top of one another "Pearls" of ectoderm are swallowed in the chronic inflammatory tissue Occasionally whole areas of ectoderm with a caseous center are engulfed, with ectodermal cells palisaded around the edge

Changes in the embryo itself are minor A few small hemorrhages may occur The epithelium of the bronchioles in the lung may be a little irregular, but destruction is usually not great and inflammatory reaction is absent (Fig 1) The occasional moribund embryo shows widespread thrombosis and hemorrhage similar to equine encephalomyelitis in the embryo Burnet (16) has described severe damage to the embryo lung following intra amniotic inoculation of human influenza virus, but he also emphasizes the lack of find ings after inoculation directly on the chorioallantoic membrane

Infection with Hemophilus

Gallavan and Goodpasture (13) found that *Hemophilus pertussis* was capable of reproducing the pulmonary lesions of whooping cough when inoculated into the amnion Later (14) it was found that strains of *H influenzae* isolated from cases of meningitis frequently caused septicemia and occasionally a meningo-encephalitis in the embryo The exact relation of these organisms to the *H influenzae suis* here studied, is difficult to determine.

Two more types of *Hemophilus*, even further removed, have been studied in the embryo *Hemophilus ducreyi*, the cause of chancroid, induces characteristic lesions in the embryo but cannot readily be carried for more than a few generations. The organisms may be found in large clumps in the infected tissue (15) *Hemophilus gallinarum*, the cause of acute coryza of chickens (26), will produce septicemia in embryos 12 to 13 days old when inoculated into the amnion Masses of the bacteria may be found both intra- and extracellularly in the infected lung tissue (Unpublished experiments.)

It is noteworthy that the first three all produce a disease pattern resembling the natural disease The last, a natural disease of chickens, produces a much more extensive and severe disease in the embryo, for septicemia and pneu monia caused by *H gallinarum* do not occur in the adult chicken (26)

H influenzae suis kills only a small proportion of 10 day embryos, and the embryos usually throw off the infection in a few days. Heart blood cultures of thirteen 10 day embryos taken at various intervals have all been negative Pathological changes in the membrane and embryo are slight. The chorioal lantoic membrane is infiltrated with a few polymorphonuclear cells which are usually concentrated in a layer just below the slightly thickened ectoderm They may be grouped together in nodules Inflammatory changes are never as marked as in the virus embryos and are practically absent from the central part of the mesoderm It is difficult to find any bacteria in the sections. A few perivascular foci of polymorphonuclears are occasionally seen in the embryo proper These have also been noticed in chick embryos infected with human meningeal strains of *H influenzae* (14) Older (15 to 19 day) embryos are usually not susceptible to *H influenzae suis*, although 2 of 12 heart blood cultures from 16 day old embryos were positive

The Combined Infection on the Membrane

Sections of membranes with the complex infection show the same basic pattern as do sections procured after inoculation of the individual components, but the changes are usually more marked Great masses of mononuclear cells are found around the vessels of the mesoderm Polymorphonuclears are scattered everywhere In addition there is frequent thrombosis of the blood vessels, with necrosis of the surrounding tissues If an ulcer such as those described in Table V is sectioned, the destroyed and necrotic tissue may be seen pushed out onto the surface of the ectoderm There are masses of *Hemophilus* deep in the base of the ulcer, mostly in the form of short rods If the embryo survives the infection and recovery sets in, the same general picture described for late infections with the virus alone is seen

The Combined Infection in the Embryo

The salient histopathological features of the natural disease in the pig are a plugging of small bronchi and bronchioles with polymorphonuclears, a destruction of the bronchial cilia, an extensive peribronchial round cell infiltration, and a collapse of the alveoli with desquamation of the epithelium (1) All of these are reproduced in the chick embryo (Figs 2 and 3) except destruction of cilia, which are not present until the 14th day A high mortality has been demonstrated in embryos inoculated on the chorioallantoic membrane with the virus and bacterium of swine influenza If surviving embryos are studied several days after inoculation, we find a remarkably selective destruction of the embryo lungs The epithelium of the smaller parabronchia and their adjoining saccules has frequently sloughed off into the lumen, the parabronchia themselves have collapsed and are later virtually obliterated by inflammatory tissue

The perivascular inflammatory reaction, which is represented in a few scattered foci in embryos receiving *Hemophilus* alone, spreads extensively throughout the lung in the complex infection Mononuclear cells now predominate near the bronchioles With the collapse of the parabronchia and bronchioles the whole lung becomes completely overwhelmed by inflammatory tissue so that only a suggestion of the original structure remains (Fig 3) The skeletons of the parabronchial walls are surrounded and infiltrated by both polymorphonuclear and mononuclear cells Polymorphonuclears penetrate into the center of the desquamated epithelial mass (Fig 8) The larger bronchioles are plugged with a polymorphonuclear exudate Moderate inflammatory changes may even develop in the tubular connections between the embryo lung and the air sacs The sinuses may also be filled with a similar exudate (Fig 7) although the destruction of the epithelial lining of the sinuses, like that in ferrets given virus alone (27), is at least not invariably present

No other organs have shown pathological changes

It was earlier pointed out that killed cultures of *Hemophilus* could be substituted for the live cultures and would still kill embryos infected with swine influenza virus. Histological examination of an embryo receiving the combination of virus and killed bacterium shows that the killed bacteria will stimulate an outpouring of polymorphonuclears into the parabronchia and larger bronchioles (Fig 6)

The histological description of these changes is based on examination of sections from 29 embryos, of which 8 received the combination of agents A more extensive series will be necessary for an accurate description of the pathogenesis of the combined infection This study, however, establishes the fact that the complex infection is entirely different pathologically from the infections produced by either agent alone

DISCUSSION

The chick embryo is being used more and more frequently for the study of bacterial and virus infections because, as Goodpasture (28) recently stated, "it seems to have little or no natural immunity of cell types ordinarily susceptible to particular viruses or bacteria in the usual hosts, at least until the last few days of incubation. At certain stages the embryo seems to offer in a way very similar to the natural host specifically favorable environments for the infectious agent."

The present study demonstrates that these statements are also true for a complex infection, caused by a combination of bacterium and virus The combined infection of the embryo has a mortality several times that of the sum of the individual components This synergism also has the same specificity that is present in the pig Finally, the histopathological response mimics the natural disease, for the combined inoculation of the membrane produces a selective destruction of the embryo lungs, thus emphasizing the pneumotropic qualities of the combination

It is true that Burnet (16) has demonstrated that the virus of human influenza will produce a profound destruction of the embryo lungs *when inoculated into the amnion*, but this type of inoculation admittedly allows the virus immediately to gain access to lung tissue Inoculation of the chorioallantoic membrane with the swine virus produces lung destruction only if cultures of *Hemophilus* are added

Only the most tentative and hesitant explanations of this phenomenon can at present be suggested. The swine virus is present in the embryo following chorioallantoic inoculation, even though the changes so produced are minor The addition of cultures of *Hemophilus* in some way brings out the pathogenic properties of the virus This may occur by means of some bacterial toxin The lack of bacteria in the embryo lung proper and the action of the killed bacteria would suggest this. But this cannot be the complete explanation, for

we have demonstrated that infection of embryos with the virus allows the *Hemophilus* to persist longer and to produce larger ulcers

It would seem that the establishment of the synergistic effect of *H influenzae suis* and swine virus in the embryo furnishes us with a tool wherewith to study the combined effect of similar agents isolated from human pandemic influenza With its aid the hypothesis of a complex etiology of human pandemic influenza may be more adequately tested

SUMMARY

The synergistic effect of *Hemophilus influenzae suis* and swine influenza virus in the pig can be reproduced by the inoculation of these agents on the chorioallantoic membrane of 9 to 10 day old chick embryos Two strains of human influenza virus that were studied failed to substitute for the swine virus in the synergistic reaction No loss of synergistic effect was noted when the swine influenza virus was put through 11 chick embryo passages Recently isolated and old stock strains of *Hemophilus* were equally able to enhance the effect of the virus Heat-killed cultures of *H influenzae suis* can be substituted for the bacterial component of the reaction Infection of the embryo with swine influenza virus predisposes to infection with *H influenzae suis*

The combination of *H influenzae suis* and swine influenza virus causes a selective destruction of the embryo lungs, not produced by the individual components This pneumonia exhibits the essential features of the natural disease

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EXPLANATION OF PLATES

The sections were stained with hematoxylin and eosin

The photographs were made by Mr J A Carlile

Plate 1

Fig 1 Lung from 13 day embryo inoculated on chorioallantoic membrane when 9 days old with swine influenza virus × 112

Fig 2 Lung from 12 day embryo inoculated 3 days previously with swine influenza virus and 36 hours previously with a culture of *Hemophilus* Both inoculated on membrane × 112

Fig 3 Lung from 13 day embryo inoculated 4 days previously (9 days) with swine influenza virus and 3 days previously with a culture of *H influenzae suis* Note complete destruction of bronchi and normal lung structure × 112

THE OCCURRENCE OF MUCOID POLYSACCHARIDE IN HEMOLYTIC STREPTOCOCCI OF HUMAN ORIGIN

By C. V SEASTONE, M.D

(*From the Department of Bacteriology, The University of Wisconsin Medical School, Madison*)

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It has been shown by Kendall, Heidelberger, and Dawson (1) that hyaluronic acid is one of the main components of the hemolytic streptococcus capsule. This polysaccharide was originally described by Meyer and Palmer (2, 3) in vitreous humor, umbilical cord, and synovial fluid, and it has since been isolated from other sources, namely skin (4, 5), a human mesothelioma (6), and the Rous sarcoma (7) Attempts to produce antibodies against this substance have so far been unsuccessful and there would seem to be little possibility that specific serologic reactions can be used for its identification

Hyaluronic acid will unite with normal serum proteins and egg albumin at about pH 4 to produce an insoluble complex. This property originally noted by Meyer and Palmer (2) has been employed in a variety of ways (8, 9) to estimate the material, it can exclude the presence of as little as 2 gamma per cc. with certainty, although it is obviously not a specific reaction

Enzymes capable of hydrolyzing hyaluronic acid, incidentally destroying the property of union with protein at pH 4, have been obtained from pneumococci (10, 11), *B welchii* (10), testicular extract (12, 13) leech extract (4, 13), and from a few non mucoid hemolytic streptococci (9, 13) Such enzymes used in conjunction with the protein reactions noted above will give a fairly accurate impression of the presence and amount of the polysaccharide in impure mixtures.

The purpose of the present study, based on the foregoing facts, was to deter mine the occurrence of hyaluronic acid, or mucoid polysaccharide, in a number of beta hemolytic streptococci obtained from human infections, and from nor mal throats in which no subsequent infection developed

Materials and Methods

125 strains were investigated, of these, 90 were from the Wisconsin General Hospital and Student Infirmary [1] obtained over a period from November, 1940, to May, 1941, these organisms presumably derived from infectious processes 35 strains were from routine throat cultures of children admitted to the Children s Hospital at Iowa City [1] In none of the latter group was any infection noted after admission.

In addition to the mucoid polysaccharide (henceforth referred to as MP) estima

[1] We are indebted to the staff of the Wisconsin State Laboratory of Hygiene for its cooperation in obtaining the hospital strains, and to Miss Marion Jones, University of Iowa Medical School, for sending us the normal throat strains

tions, the Lancefield group and encapsulation of each strain was determined. The clinical records of the patients furnishing the 90 hospital strains were examined to confirm as far as possible the presence of infection as well as its severity.

Isolation of Strains.—Pour blood plates were made with saline suspensions of the throat or wound swabs, and beta hemolytic colonies picked from these to streak blood plates to determine purity. This second plate was the source of organisms for the subsequent procedures, and except as noted, no further passage on artificial medium was carried out. The pour plate is desirable since 21 of the strains showed beta hemolysis only when under the surface of the agar, giving the alpha change on the surface. This was confirmed on the secondary streak plates by incising the agar in the heavy portion of the streak thus pushing the organisms beneath the surface. Under these conditions strong beta hemolysis was observed, as in the original plate. The majority of the non-group A strains exhibited this behavior, although 14 of the 21 strains were in group A. The 35 normal throat strains were from triplicate streak plates incubated aerobically, in 10 per cent CO_2, and anaerobically.

Method for Quantitating the Mucoid Polysaccharide.—In working out a method for determining mucoid polysaccharide (MP) based on the acid protein reaction, simplicity and speed were sought since the method was to be applied on a rather large scale. The material can be obtained either from neutralized 24 hour culture supernates, or from very young capsulated organisms. On heating such young organisms the polysaccharide is promptly released into the solution.

At first a one-tube method was investigated in which the turbidity developing in mixtures of culture supernates and acidified buffered serum was compared with $BaSO_4$ standards. Reproducible results could be achieved only with extraordinary attention to details of pH, salt concentration, and composition of the medium. Since it was felt that the method would not be generally practicable, it was abandoned.

Another method which was followed for a time was based on the observation that young capsulated organisms would agglutinate very vigorously when mixed with equal amounts of 0.5 M acetic acid. That the agglutination was very likely due to the interaction of MP and protein was indicated by the following where it appeared that the bacterial body was furnishing the protein component. 24 hour (spontaneously decapsulated) streptococci also agglutinated in 0.5 M acetic acid, but the reaction was abolished by 4 washings in distilled water; this did not occur with the young capsulated form. Resuspending the old washed organisms in either the original supernatant broth or in purified vitreous humor hyaluronic acid solution restored the agglutination. No reaction occurred in 0.5 M phosphate buffer at pH 7. The mechanism of the young capsulated organism flocculation was not immediately apparent since the surface exposed in this case would be preponderately polysaccharide. However it was found that under such conditions of acidity the MP is very rapidly released from the cell with an associated disappearance of the capsule, the situation then becoming similar to that of the old culture. A sufficient number of non-MP producing strains exhibited acid agglutination to invalidate any general application of this as a method.

The procedure finally adopted consisted of serial dilutions of neutralized culture supernatants layered over acidified normal serum, precipitation taking place at or above the interface. The medium employed was bacto-neopeptone 1 per cent, bacto-

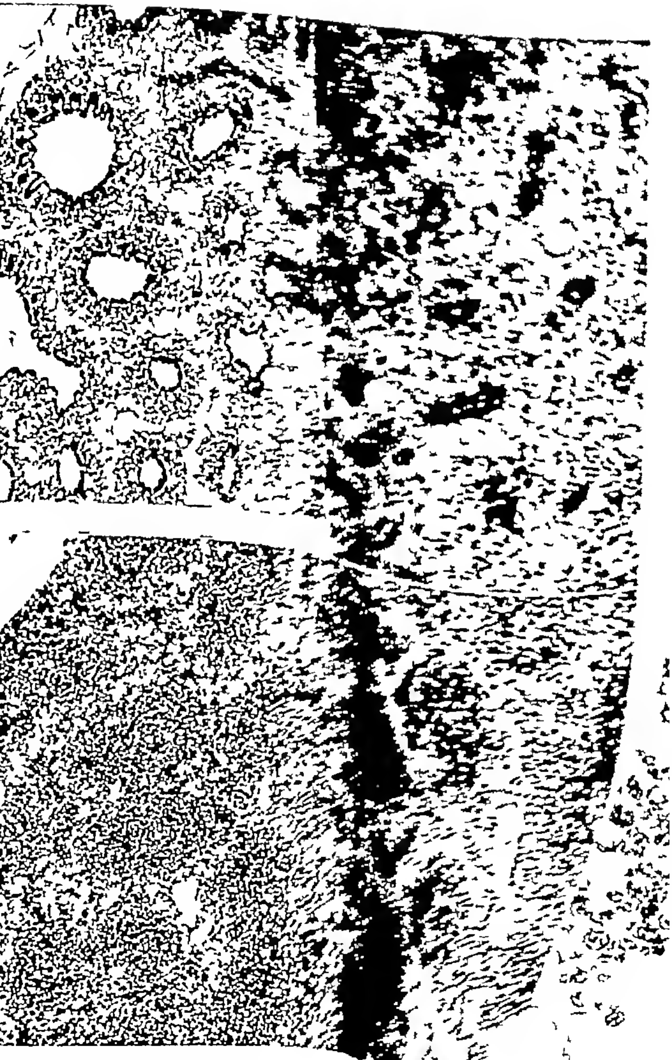

Plate 2

Fig 4 Chorioallantoic membrane of 12 day embryo infected 2 days previously with swine influenza virus × 117

Fig 5 Chorioallantoic membrane of 13 day embryo infected 3 days previously with swine influenza virus × 47

Fig 6 Lung from 13 day embryo inoculated 4 days previously with swine influenza virus and 3 days previously with killed culture of *Hemophilus* Note leucocytes in bronchi × 286

Fig 7 Polymorphonuclear exudate in sinuses of 12 day embryo infected 3 days previously with swine influenza virus and 36 hours previously with a culture of *Hemophilus* Both inoculated on membrane (Same embryo as in Fig 2) × 159

Fig 8 Remnants of bronchus in embryo given combination of swine influenza virus and *H influenzae suis* Enlargement of Fig 3 × 804

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beef extract 0 5 per cent dextrose 1 per cent NaCl 0 5 per cent, and sterile sheep serum 10 per cent, distributed in 5 cc. amounts in centrifuge tubes Inoculations were made from the second blood plates noted above. After 24 hours incubation at 37°C, 2 drops of 0 02 per cent phenol red were added and the culture was brought to about pH 7 6 with 0 5 M NaOH This neutralization is essential since sufficient acid may be developed during growth to cause complete MP precipitation, either with the bacterial or serum proteins After centrifugation of the neutralized culture serial dilutions of the clear supernatant medium in physiologic salt solution were made, 1 10 1 20 1 40 and 1 80 These dilutions as well as the undiluted supernatant, were run into micro precipitin tubes over acidified normal horse serum and readings made after ½ hour The acid serum reagent was prepared in the following manner Clear non hemolyzed horse serum was diluted 1 10 with 0.5 M acetate buffer at pH 4.2 The reaction was brought to pH 3 1 with 4 M HCl and merthiolate 1 1,000 added to a final concentration of 1 100 000 It should be noted that colorimetric (brom cresol green) pH determinations in the presence of this amount of protein are extremely inaccurate, however no significant difference in titre was noticed in a pH range between 3 and 4 Uninoculated medium gave no ring at the interface. The acidified serum may be stored in the cold for many months

Cultures reacting in a dilution higher than 1 80 have not been encountered Using purified hyaluronic acid from bovine vitreous humor an end point of comparable intensity is obtained with a solution containing 0 002 mg per cc. This would indicate the presence of about 0 16 mg per cc. of culture supernatant in a strain producing maximal amounts, a figure of the same order of magnitude has been found for group C strains by means of a somewhat more accurate method (8) as well as for group A strains by direct yield (14)

Evaluation of MP Estimation Method—Although it is perhaps unlikely that any of the recognized streptococcal somatic elements would be liberated during growth in large enough concentration to precipitate under the conditions of this method, the question of its specificity naturally arises. It will be shown later that the majority of strains possessing acid serum reactivity fall into Lancefield's group A, which might point to the C substance as the responsible factor With this possibility in mind, formamide extracts (15) were prepared with A strains giving 1 80 acid serum titres, and from A strains in which no reaction appeared. These extracts were tested with a potent A grouping serum, with the result that the latter non MP producing strains reacted in slightly higher dilutions than did the former strong producers. Furthermore neutralized culture supernates failed to give precipitin reactions in any dilution when tested with group A specific serum

The M substance of Lancefield was also to be considered in the light of its known acid precipitability The following findings tend to eliminate this and the C substance as sources of error Neutralized (pH 6 in this case) culture supernates were prepared from 31 strains, including all degrees of acid serum reactivity Treating these with hyaluronidase from a Type I pneumococcus (10 11) either eliminated completely, or reduced to a faint trace the acid serum precipitate in all but one of the

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[2] About 1 inch long from tubing 3 to 4 mm. inside diameter Such tubes are used only once.

31 tested This one reacted in a dilution of 1 10 before and after enzyme treatment From the foregoing it may be concluded that in almost all instances the estimation method given here is reasonably reliable

Encapsulation —Before neutralization of the 24 hour culture, 0 2 cc were inoculated into 2 0 cc of the medium described above in which 50 per cent defibrinated sheep blood was substituted for the serum After incubation for 3½ hours at 37°C, Wright's stained films were prepared

Lancefield Grouping —The formamide micro method described by Fuller (15) was used on organisms from serum-free broth

FINDINGS

In addition to MP determinations, Lancefield grouping, and capsule stains, the type of colony appearing on freshly prepared neopeptone blood agar as recommended by Dawson, Hobby, and Olmstead (16), and the nature of the growth in 10 per cent serum broth were noted Streptococci failing to produce the MP factor almost invariably remained glossy However, among the strains producing this material, many were encountered which also showed no disturbance of the colonial surface Furthermore in those strains showing flattening, crater formation, or roughening of the surface, one could not correlate the degree of this change with the amount of MP produced

Concerning the growth in 10 per cent serum broth, there was a distinct tendency for the non-producing strains to show an extremely granular type of growth, while the MP producers remained uniformly suspended These observations were made on neutralized cultures to eliminate the effect of MP-protein precipitates In our particular group of strains this could be regarded only as a tendency, the most common exception being the frequent occurrence of non-MP producing strains which grew as very uniform suspensions under these conditions

The results appear in Table I from which several facts may be demonstrated All the strains showing positive reactions in the 1 40 to 1 80 range of dilution may be shown to be capsulated In the 1 20 range, capsules appear irregularly, and below 1 20 no stainable capsule could be found In general, the largest capsules appeared in the 1 80 group but it was not always possible to correlate capsular size with MP titre Different strains retain their capsules with varying degrees of tenacity, and exhibit different growth rates It would therefore be unwise to assume that any one arbitrary incubation period would allow optimal capsulation of all strains Of the 42 non-group A strains,[3] not one was found to produce detectable amounts of mucoid polysaccharide The distribution of group A strains in the hospital compared with the normal throat strains is noteworthy, confirming the well established observation of Lancefield (17) that most streptococcal infections in man are due to this group Of the

[3] These were in groups B and C with one exception which could not be classified

35 strains from normal throats only 3 were MP producers, all of them capsulated and belonging to Lancefield's group A. No other group A strains were encountered here In sharp contrast are the 90 hospital strains, if one disregards the 8 strains which were not associated with infection, 78 of 82 strains are group A, and 72 are MP producers.

In evaluating the severity of the infections it is fully recognized that the virulence of the organism is not the only factor involved. Of perhaps equal importance in affecting the clinical picture is the individual host resistance as well as the sulfonamide therapy which most of the patients received In

TABLE I

Distribution of Mucoid Polysaccharide

Titre in acid serum	1:40 to 1:80	1:20	1:10	Undiluted or 0
90 hospital and infirmary strains				
No of strains	51	15	6	18
Showing capsules	51	12	?1	?1
Lancefield group A	51	15	6	6
From severe infections	14	2	1*	0
From moderate infections	29	11	4	5
From mild or doubtful infections	8	2	1	5
Giving no clinical evidence of infection	0	0	0	8
35 normal throat strains				
No of strains	2	1	0	32
Showing capsules	2	1		0
Lancefield group A	2	1		0

* Complicated by *Staphylococcus aureus*

spite of these limitations the infections were grouped as severe, moderate, and mild It was of interest that a certain number of the strains were derived from patients showing no clinical or pathological evidence of streptococcal infection.

The group of severe infections comprised those showing a septic febrile course, a positive blood culture, or a particularly protracted illness with or without a spiking temperature curve. All exhibited marked leukocytosis. Four of these infections were fatal, and one required amputation of a limb In the moderate group were placed infections of rather shorter duration and less alarming nature. Most of these were acute sore throats with more or less intense pharyngeal injection, and a 3 to 5 day temperature elevation, often followed by otitis media It is not certain that all of the so called mild cases were of streptococcal etiology They were usually transitory sore throats with a 24 to 48 hour febrile period sometimes without leukocytosis or marked pharyngeal

inflammation One of them was an otitis media in a diabetic child aged 7, who remained afebrile throughout the course of the illness

As is shown in the table, there is a progressive decrease in the proportion of high MP-producing strains as one goes from the severe types of infection to the mild ones No fatalities occurred due to strains precipitating in a titre of less than 1 40 Although the numbers were too small to have much significance, a relatively greater proportion of children were infected with organisms of low MP content

DISCUSSION

The data presented above tend to confirm the impression that most serious human hemolytic streptococcal infections are due to organisms in a phase described by Dawson (16) as mucoid, and by Todd and Lancefield (18) as matt, these designations very likely being synonymous The important factor in the matt designation has been the M substance, while the common factor indicated by Dawson and others as associated with mucoid strains is the mucoid polysaccharide, or hyaluronic acid, presumably a capsular substance The latter has not been sought in any very large number of human strains, and it is not impossible that these two substances are invariably associated in any matt, or mucoid strain The present work shows the wide distribution of the mucoid polysaccharide in streptococci from man, and the wide variation in the amounts appearing in different strains In the absence of a more or less quantitative study, many of these strains, although producing appreciable amounts of polysaccharide, would not ordinarily be classified as mucoid, indeed they could not under the current description of this phase

Lancefield has shown that the protein M substance is partly responsible for type specificity in group A streptococci, and for the protective effect of antisera and vaccines There is evidence that the anti-M antibody brings about opsonization of capsulated organisms, this has been suggested as a typing method (19) These facts would constitute good evidence that the M substance is responsible for virulence were it not for the observation that this material may be obtained in equally large amounts from avirulent strains (20) That one might stimulate protective antibodies with a substance not itself responsible for virulence is conceivable, recalling the non-type specific immunity obtained with the pneumococcus (21)

The possibility that the mucoid polysaccharide may play a part in virulence is raised in the first place by its capsular location, the fact that capsular autolysis is associated with the appearance of spontaneous phagocytosis in normal blood is also suggestive (22) Hirst (14) has studied the effect of leech hyaluronidase on protection He was able to show that this enzyme which causes rapid decapsulation in strains of either group A or C, is protective only for mice infected with group C strains, and it was concluded from this (23) that the

capsular substance plays little or no part in the virulence of group A strains. Using another source of hyaluronidase, an extract of beef testis, we have obtained essentially the same results with the same strains employed by Hirst, however it has been our experience that group C organisms of guinea pig origin are less rapidly fatal than are group A strains The average time of death for the latter is around 24 hours, while the fatal period for group C infections is nearer 48 hours With this in mind as a possible explanation for the differences in therapeutic effect, we repeated the experiment using larger amounts of enzyme at more frequent intervals, and obtained permanent protection in group A (S 23) infections involving between 10 and 100 M.L.D This effect is mentioned only in a preliminary way for the purpose of discussion, more detailed study is in progress

These *findings point* to the *mucoid polysaccharide* as a *factor* of *definite* significance in the virulence of hemolytic streptococci of human origin.

SUMMARY

1 A rapid method for the roughly quantitative estimation of mucoid polysaccharide in hemolytic streptococci has been described

2 Using this method, about 94 per cent of strains from moderate or severe streptococcal infections in man have been found to produce mucoid polysaccharide in greater or less amount In a group of streptococci from normal throats only about 8 per cent produced this substance, all of the producers falling into Lancefield's group A

3 Of the Lancefield group A strains from both normal and infected sources, 92 per cent showed the presence of mucoid polysaccharide in culture dilutions of 1 10 or higher

4 The probable significance of the mucoid polysaccharide in streptococcal virulence is indicated

We are indebted to Mr E H. Kass for much assistance in this work, particularly in connection with the enzyme preparations.

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f Bacterial Growth —Since the number of organisms in a clear ional to the opacity of the suspension, bacterial growth can be dity (21) In these experiments measurements of turbidity were man universal spectrophotometer, the readings on the logarithmic ed to and expressed in terms of optical density (turbidity) To comparing the growth curves thus obtained with those produced t should be emphasized that turbidimetric methods measure the organisms, both living and dead, while plate counts measure only iable organisms. A tube of uninoculated basal medium was used he colorimeter

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ation of Growth Curve during Development of Resistance to Constant Concentrations of Sulfonamides

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re shown graphically in Fig 1 Following the initial lag period, inhibition of growth the 1st day in all the tubes containing The greatest difference in growth between the control and occurred from the 4th to the 6th hour after inocula a gradual daily development of resistance, the progress of

lococci were found to be resistant to all five drugs after being transferred nine times in sulfapyridine and sulfathiazole (8) With the gonococcus, the failure of one group to demonstrate insensitiveness to sulfathiazole (16) has given rise to the clinically important editorial suggestion that "the failure of the gonococcus to develop resistance to sulfathiazole suggests that sulfathiazole-fast strains are not likely to be developed in the clinic or to be spread to the general population" (17) Other investigators (18), who have demonstrated sulfathiazole-resistant gonococci, have naturally challenged this statement (19)

These discrepancies are probably more apparent than real, and are due to technical differences in the methods of performing the experiments Among the important variables are the nature of the medium, the size of the inoculum, the concentration of sulfonamide, and the method of measuring the results For example, a given inoculum of an organism may apparently be resistant to 100 μg per cc of sulfanilamide and not to a similar concentration of sulfathiazole simply because the latter drug is so many times more potent than the former that the method of measuring the results is not sufficiently sensitive to record the small degree of resistance that is actually present

The purpose of the present paper is to describe a method for studying the quantitative aspects of the resistance of an organism to various sulfonamides, and to present the data obtained when this method was applied under rigidly controlled conditions It was hoped that through such a study the discrepancies of the earlier work might be clarified, and that a clearer knowledge of the fundamental nature of the development of sulfonamide resistance might be obtained

Method and Materials

Culture Medium —A synthetic medium, which was employed for all experiments, was prepared in the following manner —

$(NH_4)_2SO_4$	5 gm
NaCl	5 gm
Glucose	2 gm
KH_2PO_4	3 gm
Casamino acids Difco	2 gm
Distilled water to 1000 cc.	

The pH was adjusted to 7 6 with 1 N NaOH, and the solution was distributed in 8 cc amounts into 22 by 175 mm test tubes and autoclaved at a pressure of 15 pounds for 15 minutes After incubation overnight to test for sterility the medium was stored in the ice box.

Organism —A strain of *E coli*, isolated from the blood stream of a patient with pyelonephritis and bacteremia, was selected for the experiments, and was transferred daily in the basal medium This organism showed typical cultural reactions as defined by Bergey (20)

Reagents —Sulfanilamide, and the sodium salts of sulfapyridine, sulfathiazole,

and sulfadiazine, were dissolved in distilled water in the following concentrations 100, 50, 10, 5, and 2 5 mg per 100 cc. The solutions were heated in the Arnold steamer for 20 minutes, and were stored in the ice box.

Determination of Bacterial Growth—Since the number of organisms in a clear medium is proportional to the opacity of the suspension, bacterial growth can be measured by turbidity (21) In these experiments measurements of turbidity were made with a Coleman universal spectrophotometer, the readings on the logarithmic scale were converted to and expressed in terms of optical density (turbidity) To avoid confusion in comparing the growth curves thus obtained with those produced by plate counts, it should be emphasized that turbidimetric methods measure the total number of organisms, both living and dead while plate counts measure only the number of viable organisms A tube of uninoculated basal medium was used for the blank in the colorimeter

Inoculum—The inoculum was designed to produce a range of turbidity which could be accurately measured with the spectrophotometer Since the measurements were quantitative, every effort was made to inoculate a constant number of organisms for each experiment. A 24 hour growth of the organism to be tested was diluted to an optical density of 0 1 in sterile saline. 1 cc. of this suspension was then diluted 1 10 in sterile saline, and 1 cc. of the final dilution was used for the inoculum. Plate counts showed that the final concentration of the inoculum, after addition to the basal medium, was approximately 1 million viable organisms per cc.

Preparation of Test Solutions—12 hours before each test 1 cc. of each sulfonamide solution was added to 8 cc. of basal medium The tubes were put in the incubator so that the media would all be at 37°C. when the organisms were added. This was an important step in producing constant results for the amount of growth varied widely when the organisms were added to media of different temperatures. 1 cc. of the inoculum was added to each tube, making a total of 10 cc. To substitute for the sulfonamide 1 cc. of sterile saline was added to the control tube.

Determination of Growth Curve during Development of Resistance to Constant Concentrations of Sulfonamides

Employing the technique and precautions described, the organism was transferred daily in basal medium plus the following constant amounts of sulfonamides, sulfanilamide 100 µg /cc., sulfapyridine 10 µg /cc., sulfathiazole 2 5 µg /cc., and sulfadiazine 2 5 µg /cc. These drug concentrations were selected because they all had approximately the same bacteriostatic potency Daily readings of the turbidity of the suspensions were made every 2 hours for 8 hours, and a final measurement was recorded at 24 hours.

The results are shown graphically in Fig 1 Following the initial lag period, there was marked inhibition of growth the 1st day in all the tubes containing sulfonamides The greatest difference in growth between the control and drug inhibited organisms occurred from the 4th to the 6th hour after inoculation There was a gradual daily development of resistance, the progress of

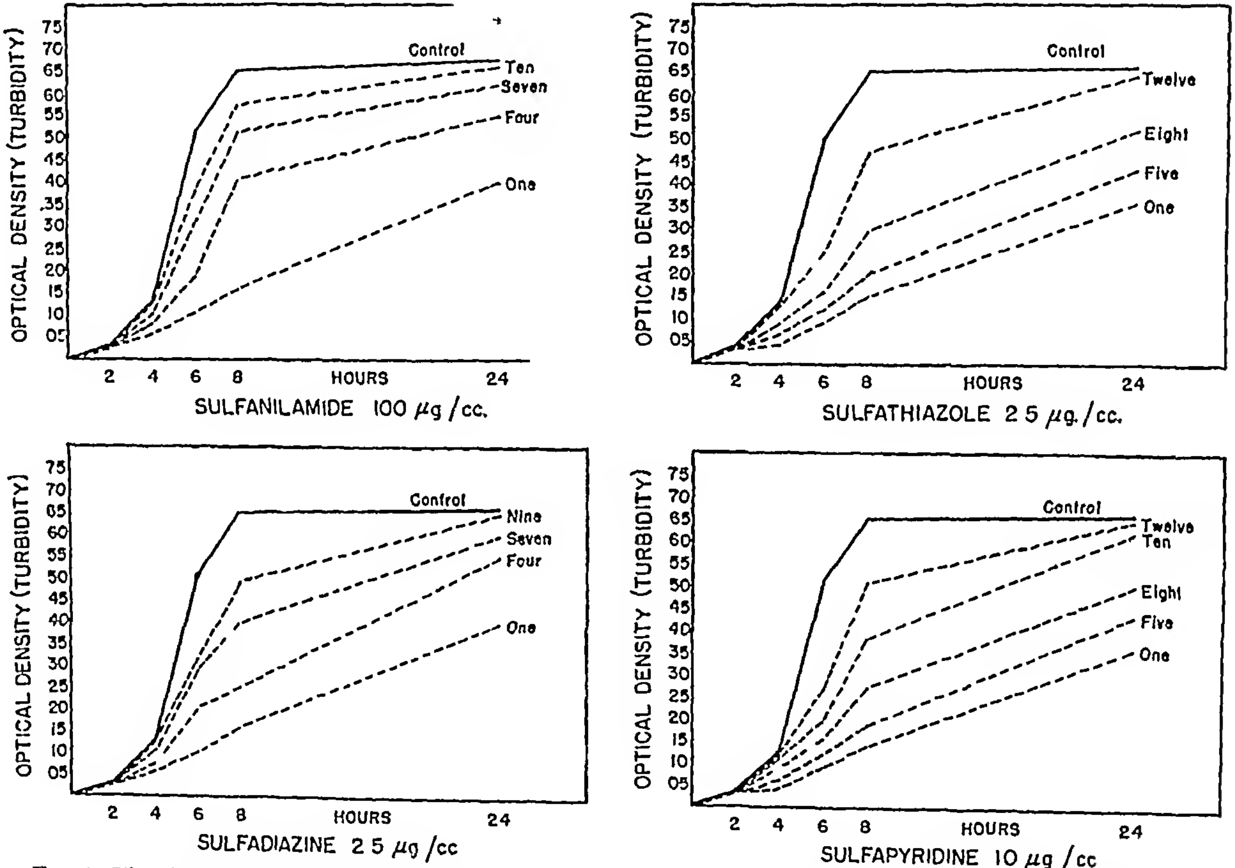

FIG 1 The development of resistance of *E coli* to four sulfonamides Constant numbers of organisms were transferred daily in basal medium plus the concentrations of sulfonamides given below each graph Resistance developed gradually for all the drugs, becoming maximal in from 9 to 12 days The number at the end of each growth curve indicates the day on which the curve was recorded

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Two points deserve special comment. One is that when maximally resistant, the organisms were in no instance totally resistant, *i.e.*, they did not grow as well as the control However, if readings were made only at the end of 24 hours, this would not be apparent The explanation for this is that with the heavy inoculum employed there was medium sufficient for only a certain amount of growth, and at the end of 24 hours both the control and resistant organisms had grown out to this extent. However, the 4 and 6 hour readings clearly show considerable inhibition of the resistant organisms Thus, the time at which the results are read is of primary importance, and erroneous conclusions may be drawn if this factor is not carefully considered The other point of interest is that the organisms became resistant to all the sulfonamides The significance of this observation will be discussed later

Determination of Quantitative Relationships between Organisms Resistant to Different Sulfonamides

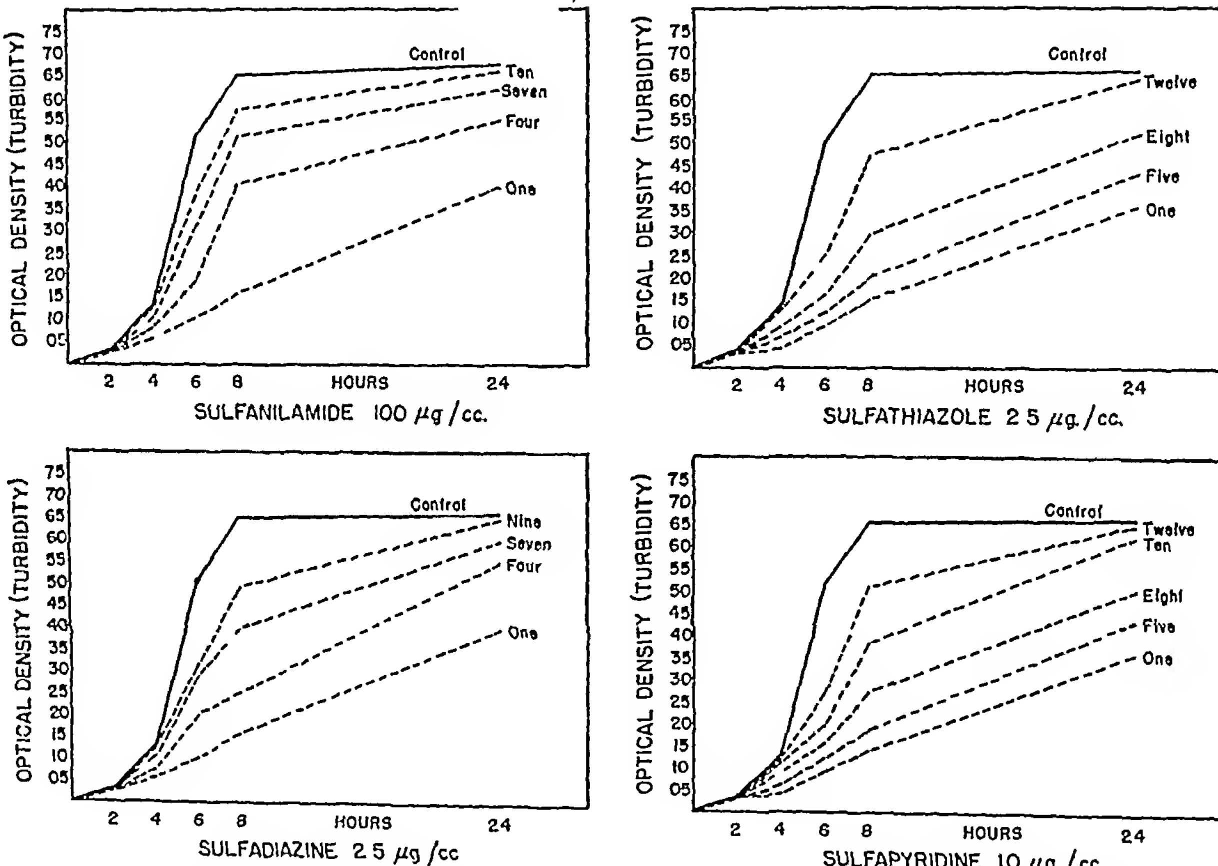
This experiment was performed in conjunction with Experiment 1 At the end of 7 days, and again at the end of 14 days, the organisms which were transferred in sulfonamide solutions, and a control organism, were set up against various concentrations of all four sulfonamide solutions in the following manner using the standard inoculum, each organism was put into tubes containing 100, 50, 10, 5 and 2.5 μg per cc. of sulfadiazine, sulfathiazole, sulfapyridine, and sulfanilamide, respectively The turbidity of the tubes was measured at 8 hours, and again at 24 hours.

Except that there was a greater degree of resistance at the end of 2 weeks, the results at the end of 7 and at the end of 14 days were essentially the same, therefore only the results at the end of 2 weeks are presented, and they are shown in Fig 2.

For the sake of clarity, the fundamental points demonstrated by this experiment are best enumerated as follows —

First, the test organism became resistant to all four sulfonamides, sulfadiazine, sulfathiazole, sulfapyridine and sulfanilamide.

Second, there was a close correlation between the degree of resistance developed and the bacteriostatic potency of each drug For example, the degree of resistance to 100 μg /cc. of sulfanilamide was the same as that developed in response to 2.5 μg /cc. of sulfadiazine, and the figures for the control indicate that these concentrations of sulfanilamide and sulfadiazine were equally effective in inhibiting the control organisms In other words, the degree of re



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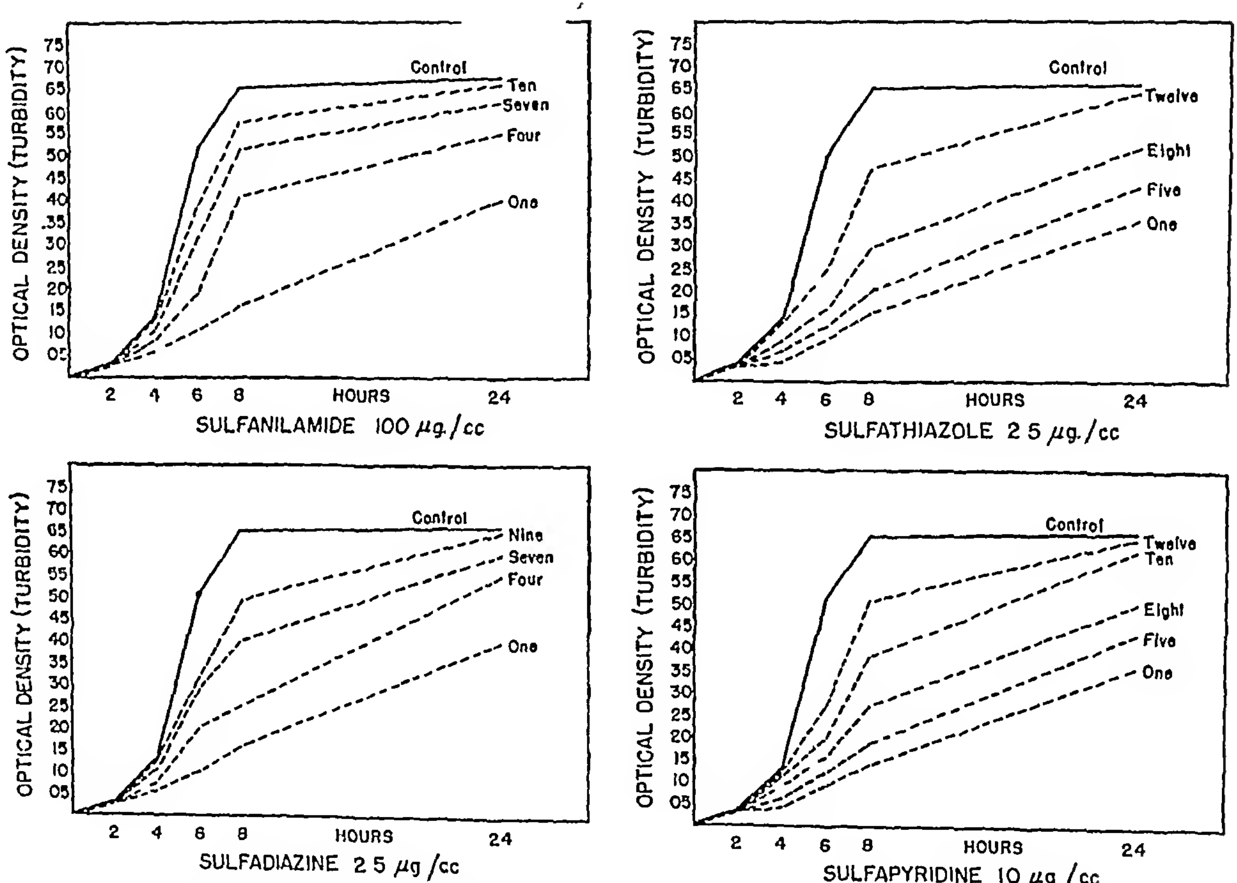

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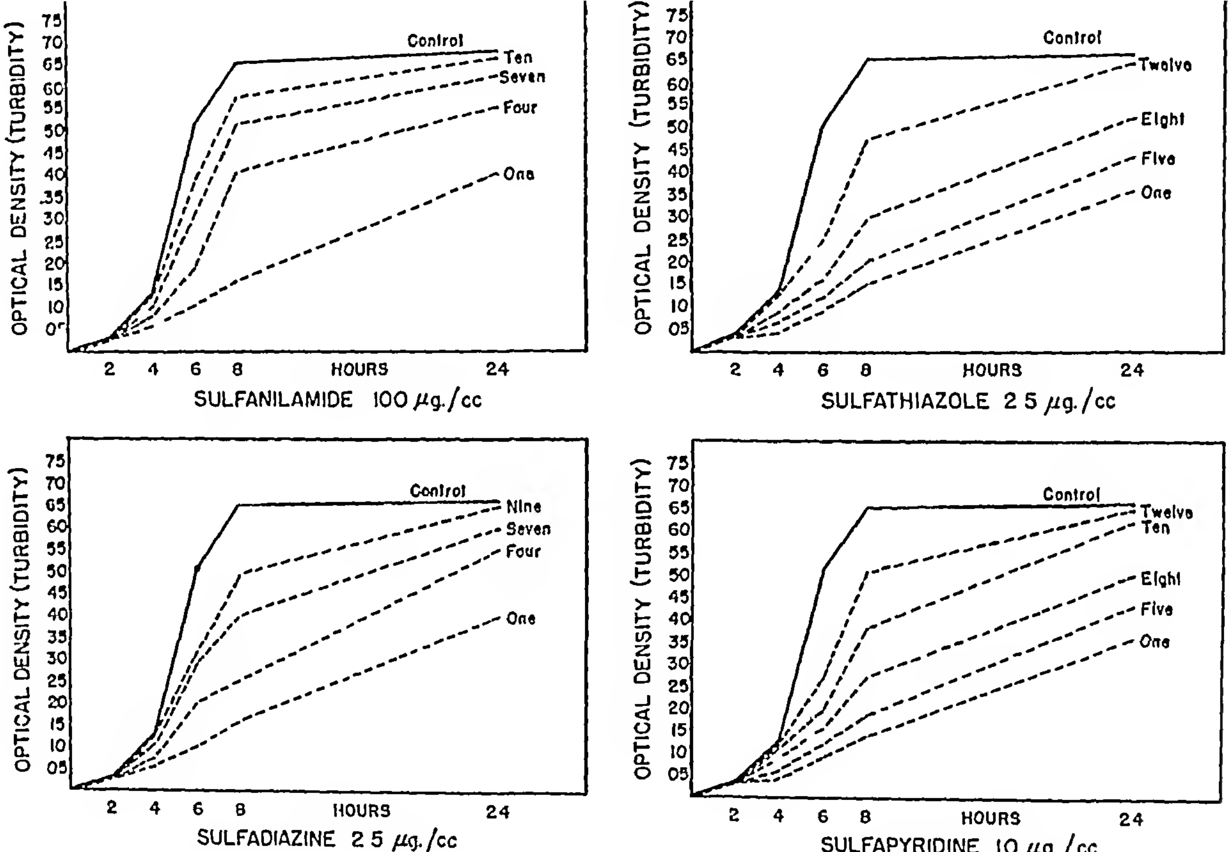

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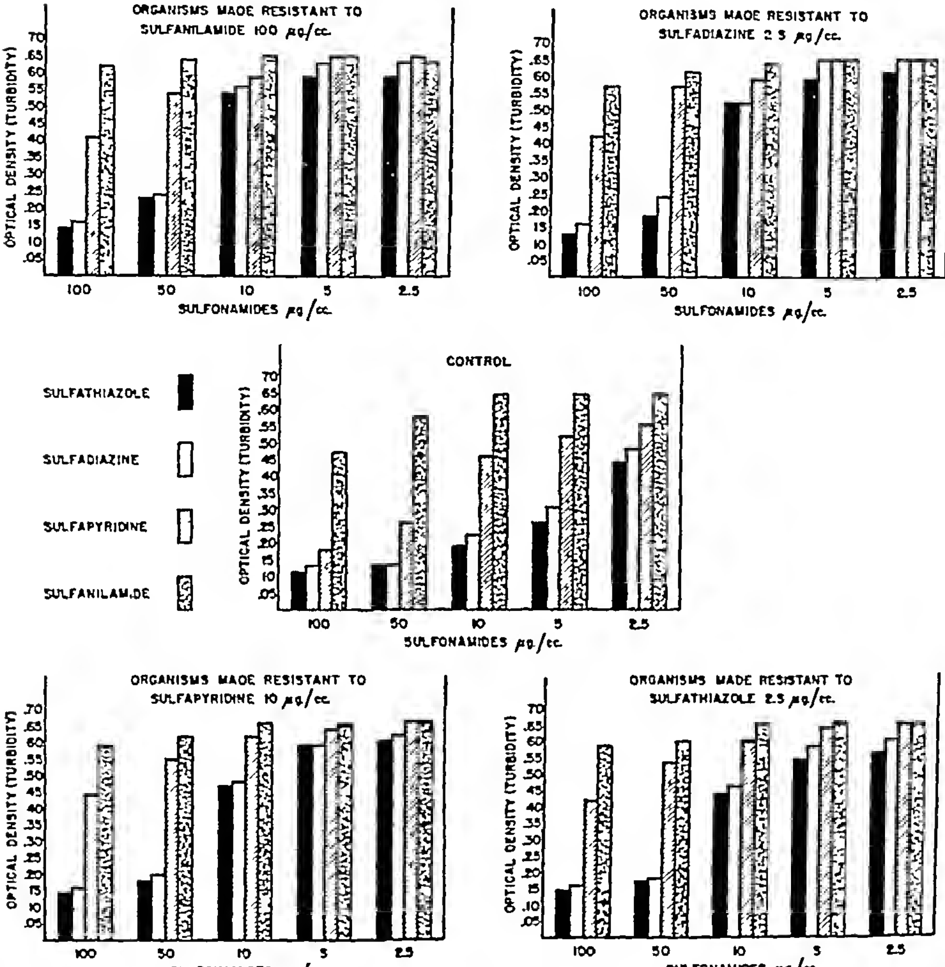

FIG 2 Quantitative relationships between organisms resistant to different sulfonamides Resistant organisms were grown in basal medium plus 100, 50, 15, 5, and 2 5 μg /cc. of sulfathiazole, sulfadiazine, sulfapyridine, and sulfanilamide The amount of growth (turbidity) at the end of 24 hours is shown above See text for interpretation of the data

Third, organisms resistant to certain bacteriostatic concentrations of one sulfonamide were equally resistant to similar concentrations of the other sulfonamides For example, the organisms made resistant to 100 μg /cc of sulfanilamide were equally as resistant to 50 μg /cc of sulfadiazine as was the

organism made resistant to 2.5 μg /cc of sulfadiazine In other words, there was no specific reaction between the organism and any one sulfonamide The same degree of resistance was developed for the heterologous as for the homologous sulfonamide The similarity in the form of all the graphs in the figure is

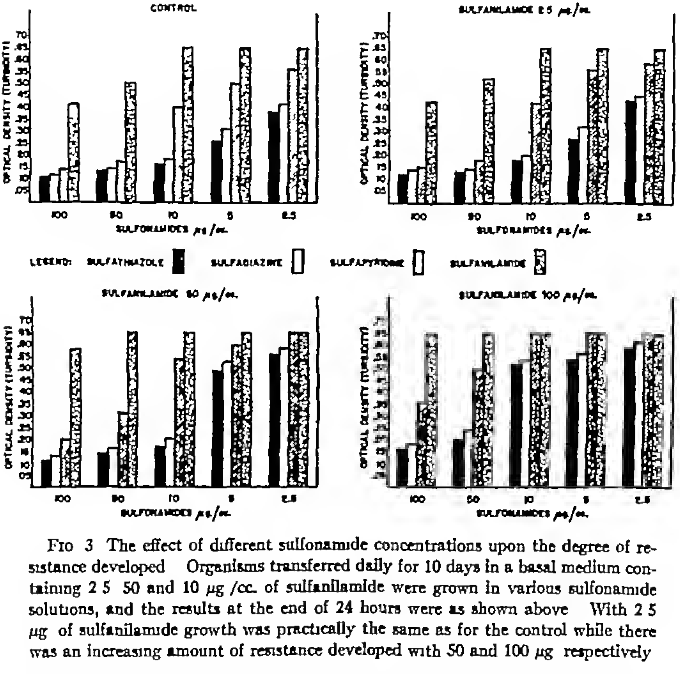

FIG 3 The effect of different sulfonamide concentrations upon the degree of resistance developed Organisms transferred daily for 10 days in a basal medium containing 2 5 50 and 10 μg /cc. of sulfanilamide were grown in various sulfonamide solutions, and the results at the end of 24 hours were as shown above With 2 5 μg of sulfanilamide growth was practically the same as for the control while there was an increasing amount of resistance developed with 50 and 100 μg respectively

a further elucidation of this point. The relative degree of inhibition of all the resistant organisms by the various sulfonamides was exactly the same in every instance as it was for the control

Fourth, organisms made markedly resistant to small concentrations of sulfonamides were only slightly insensitive to high sulfonamide concentrations To illustrate, very slight resistance to 100 μg /cc. of sulfadiazine was shown by the organism which was markedly resistant to 2.5 μg /cc. of sulfadiazine. Further the organisms which were transferred daily in 100 μg /cc. of sulfanil

amide were markedly resistant to 2 5 μg /cc of sulfathiazole and sulfadiazine, but only slightly to 100 μg /cc of sulfathiazole and sulfadiazine The effects of various concentrations of sulfonamides on the degree of resistance developed will be further clarified in the next section However, it should be emphasized at this point that the concentrations of sulfonamides used in resistance experiments are of great importance, and that some of the errors in the previous work in this field have resulted from employing concentrations of sulfonamides so great that resistance which was actually present could not be demonstrated

The Effect of Various Concentrations of Sulfonamides on the Degree of Resistance Developed

The control organism was transferred daily in tubes containing basal medium plus 100, 50, and 2 5 μg /cc. of sulfanilamide. At the end of a week the organisms were set up against various concentrations of sulfonamides in the manner described for Experiment 2

The experimental data are presented in Fig 3 The degree of resistance developed varied with the concentration of sulfanilamide in which the organisms were transferred The organisms transferred in 100 μg /cc were markedly resistant, those transferred in 50 μg /cc were moderately resistant, and those transferred in 2 5 μg /cc were only slightly resistant The slight degree of resistance developed with 2 5 μg /cc suggests that unless there is actual inhibition of growth of the organisms, the mere presence of the drug in the medium will not cause resistance to develop

To test further the effect of the concentration of the sulfonamide upon the development of resistance, organisms made maximally resistant to 2 5 μg /cc of sulfathiazole by daily transfers for 14 days were then transferred in a medium containing 100 μg /cc for 14 days As a control, the organisms were also transferred daily in 2 5 μg /cc of sulfathiazole to see if further resistance developed After 14 days the organisms partially resistant in 2 5 μg /cc of sulfathiazole had become considerably more resistant to higher concentrations of all the sulfonamides as a result of transferring them in media containing 100 μg /cc of sulfathiazole, while the control was unchanged In other words the partially resistant organisms became more resistant when exposed to an environment containing a higher sulfonamide concentration

DISCUSSION

Certain fundamental points concerning the development of sulfonamide resistance have been clarified by the quantitative experiments herein described Resistance, a gradually developing process, has been demonstrated for all four drugs tested, sulfanilamide, sulfapyridine, sulfathiazole, and sulfadiazine, regardless of their different chemical structures It has been shown that the

degree of resistance developed is directly correlated with the bacteriostatic potency of the sulfonamide, and further, that organisms made resistant to certain bacteriostatic concentrations of one sulfonamide are equally resistant to similar bacteriostatic concentrations of the other sulfonamides. These observations strongly suggest that the development of sulfonamide resistance represents an interaction between the organisms and the one common structural unit of all the sulfonamides, namely the *p*-amino nucleus.[1]

The nature of this interaction is not clear The two most widely held views concerning the nature of the development of sulfonamide resistance are (*a*) that resistant strains are the product of selective propagation of sulfonamide-resistant variants (1, 14), and (*b*) that the interaction of the drug with the organisms results in some alteration in the intermediate metabolism of the organisms, enabling them to counteract the inhibitory effect of the sulfonamides (13) The demonstration of the quantitative nature of the interaction of the organisms with the *p*-amino nucleus in the present paper strongly supports the latter view In this connection, Wood (22) has very recently reported carefully controlled experiments concerning the quantitative aspects of the inhibition of sulfonamides by para-aminobenzoic acid, in which it was shown that para aminobenzoic acid nullified the bacteriostatic effect of all six sulfonamides tested, that the bacteriostatic potency of each drug was directly proportional to its ability to counteract the antibacteriostatic action of para aminobenzoic acid, and that for different concentrations the minimum amount of para aminobenzoic acid required to prevent bacteriostasis was such that the ratio of para *aminobenzoic* acid *to drug was constant. These* observations suggested that the bacteriostatic action of the sulfonamides works mainly through the *p*-amino nucleus, which is part of the structure of para aminobenzoic acid and all of the sulfonamides, and it was felt that these data supported the hypothesis, originally advanced by Woods (23), that the antagonism of the sulfonamides by para aminobenzoic acid represented the competitive inhibition of an essential enzyme reaction by a substance chemically related to the substrate. It is of considerable interest that the observations of Wood concerning the inhibition of the sulfonamides by para-aminobenzoic acid are so similar to those recorded in the present paper concerning the development of sulfonamide resistance, and it is possible that the same enzyme system (or systems) may be responsible for both phenomena

One group has advanced the opinion that in the development of sulfonamide resistance "the actual concentration of drug employed is probably of little importance, since organisms can be made resistant to a high concentration of

[1] For the sake of simplicity the benzene nucleus of the sulfonamides with the free amino group in the para position is referred to as the 'para amino nucleus" throughout this paper

drug by transferring repeatedly in the presence of a small concentration of drug as well as by increasing the amount of drug in successive transplants" (7) The present studies indicate, however, that when quantitative measurements are made, the degree of resistance developed is greatly influenced by the concentration of drug employed Although certain limiting factors, such as the relation of the number of organisms to the total amount of medium, prevent the conclusion that a direct proportion exists, it is certainly evident that, for the concentrations employed, increasing amounts of drug caused the production of increasing amounts of resistance Conversely, it is of interest that unless the drug is present in sufficient concentration to inhibit the growth of the organisms, very little resistance is developed

A natural corollary of the evidence indicating that the *p*-amino nucleus is somehow concerned in the development of sulfonamide resistance is the opinion that all organisms susceptible to the bacteriostatic action of the sulfonamides are capable of becoming resistant to all of the sulfonamide drugs It is felt that the previous reports (7, 16) that organisms are capable of becoming resistant to some sulfonamides and not to others are the result of inadequate technical methods An attempt has been made in the present paper to control, and to point out, many of the variables which are possible sources of such errors Of considerable clinical importance is the recently widely circulated statement that gonococci are capable of becoming resistant to sulfanilamide but not to sulfathiazole This opinion has been challenged by a group who have demonstrated sulfathiazole-resistant gonococci Further, it is reasonable to assume that, in cases of subacute bacterial endocarditis, changing from one sulfonamide to another will be of no benefit to the patient once the organisms have become resistant There is considerable clinical experience in favor of this point of view The rôle that sulfonamide-resistant organisms may eventually play in human infections is uncertain, but with the evidence at hand it would seem reasonable to assume for the present that organisms susceptible to the action of the sulfonamides are capable under proper conditions of becoming resistant to all of the sulfonamide drugs

SUMMARY AND CONCLUSIONS

1 *In vitro* experiments were performed with *E coli*, using a method designed for the quantitative study of various aspects of sulfonamide resistance

2 Resistance was found to be a gradually developing process, and was demonstrated for all four drugs tested, sulfanilamide, sulfapyridine, sulfathiazole, and sulfadiazine

3 It was shown that the degree of resistance developed was correlated with the bacteriostatic potency of the sulfonamides, and that organisms resistant to certain bacteriostatic concentrations of one sulfonamide were equally resistant to similar bacteriostatic concentrations of the other sulfonamides

4 These observations were interpreted as indicating that the development of sulfonamide resistance represents an interaction between the organisms and the one common structural unit of all the sulfonamides, namely, the *p*-amino nucleus It is also suggested that this interaction may involve the same enzyme system (or systems) as those concerned in the antagonism of the sulfonamides by para-aminobenzoic acid

5 The relation of these findings to the broader aspects of sulfonamide resistance is discussed, and it is postulated that, despite reports to the contrary, all organisms susceptible to the bacteriostatic action of the sulfonamides are capable of becoming resistant to all of the sulfonamides

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THE VIRUS OF INFECTIOUS FELINE AGRANULOCYTOSIS*

I. Characters of the Virus Pathogenicity

By J T SYVERTON M.D J S LAWRENCE, M.D., R. J ACKART, M D.,‡ W S ADAMS,‡ D M. ERVIN,‡ A. L. HASKINS, Jr ‡ R. H. SAUNDERS, Jr.,‡ M B STRINGFELLOW, M.D.,‡ and R. M WETRICH‡

(*From the Departments of Bacteriology and Medicine, The University of Rochester School of Medicine and Dentistry, Rochester, New York*)

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Infectious feline agranulocytosis is a malady of cats only recently described (1-3) [1] This infection at its height can be readily recognized and differentiated from other feline diseases by blood studies which make apparent the characteristic profound granulocytopenia, a less pronounced leucopenia, and a marked relative lymphocytosis in the absence of thrombopenia and appreciable anemia. The cytological pictures of the bone marrow and peripheral blood are essentially similar to those which have been reported for human agranulocytosis. An additional characteristic pathological feature is the presence of intranuclear inclusion bodies of Type A (Cowdry) in the intestinal epithelial cells and in the reticular cells of the lymphoid tissue Detailed descriptions of the clinical, hematological, and pathological findings have been made (1-3)

It was found, early in these studies (1) that the infective agent of infectious feline agranulocytosis has properties common to characteristic viruses. However, most of the data from experiments designed to yield information about this virus were reserved for detailed presentation and now comprise the substance of two papers. The experiments described in this paper deal with the nature, properties, and pathogenicity of the virus. The second paper, which follows, presents the results of experiments that deal with the immunological relation of the virus of infectious feline agranulocytosis to other viruses. As the experimental work reported in these two papers was done concurrently and the results are mutually supplementary, these papers are presented together All materials and methods are described in the first paper The second paper includes a discussion of the facts reported in both papers, a consideration of these findings in relation to several papers which unquestionably deal with the same disease and virus (4-6), and a bibliography (the references are numbered serially throughout the two papers)

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‡ Student Fellow in Medicine

[1] The references for this paper and the succeeding paper are numbered serially throughout both, the bibliography being given at the end of the second paper

240 cats with feline agranulocytosis have been observed in this laboratory during the past 4 years The general characteristics of the disease are briefly as follows An incubation period of from 4 to 7 days (usually 5 or 6) following exposure to the infective agent merges into the stage of clinical disease, which is characterized by listlessness, inappetence, and a prone position Vomiting, diarrhea, and nasal and ocular discharges occur most irregularly Death may intervene at any time after the 5th day following exposure Oral or perianal lesions have not been noted A desire for food is the best index of recovery, which usually requires only 5 or 6 days The mortality rate approximates 50 per cent. The typical hematological changes mentioned above occur on from the 6th to 8th day after exposure. The most marked pathological findings are present in the bone marrow, lymphoid tissue, and intestinal mucosa The bone marrow when examined at the height of the disease shows a hypoplasia and an absence of differentiation of the myeloid cells The erythroid cells and the megakaryocytes are generally present in normal percentages The reticuloendothelial cells of the spleen and lymph nodes show evidence of proliferation, and intranuclear inclusion bodies (Cowdry's Type A), as evidence of specific virus activity, are not infrequently present in the cells of the gastrointestinal mucosa, the lymphoid tissues, and bronchial mucous glands

Methods and Materials

Cats—410 domestic cats were utilized for passages and to determine the presence of active virus in test and control materials They were also used in immunity tests

It became immediately apparent when we began our studies of feline agranulocytosis that the highly infectious nature of the inciting agent and the natural prevalence of the disease among cats from thickly populated districts made it essential to select and maintain each experimental animal with great precautions Accordingly, after the first 13 passages of the infective agent in series in cats (1), we limited as often as possible our supply to kittens from farms where a history of illness or death among their resident cats was denied (Even then we not infrequently encountered animals that were immune to infection by the feline virus, as some of our protocols show) Many farms, free from the disease, provided several litters each year We found that it was to our advantage to bring the animals personally from the farm to our isolation quarters in order to avoid any possibility of exposure to the infectious agent The cats on arrival were separated into groups of from 3 to 6 animals, which were maintained as entirely separate units under rigid isolation for from 12 to 92 days in rooms widely separated from the animal house and from each other Each cat was housed in a single metal cage During the period of isolation, the blood picture of each cat was followed by leucocyte and differential counts, which were made at from 1 to 3 day intervals (almost regularly every 2nd day) Many of the animals also had rectal temperatures taken Only those animals that appeared normal throughout the period of isolation were used for experimental purposes Following exposure to any material under test for infectiousness, each cat had blood studies made daily Finally, most of the animals without overt evidence of illness after exposure to the virus were subsequently tested for active immunity by the injection of a massive challenge dose of virus of known pathogenicity or by exposure to animals with the disease In

many of the experiments, 1 or 2 normal animals were maintained as controls under the same environmental conditions, but they were not handled except to have blood counts taken

At times, a single experiment, and therefore a single group of cats, answered several of the questions under investigation in this study on the nature and properties of the etiological agent Thus one experiment might yield the following information about the infective agent Its pathogenicity by a given route of inoculation, its distribution within the body of the host, and its filterability through a given candle, as determined by its pathogenicity following filtration. Accordingly, some of the 410 experimental cats employed may be referred to several times.

Other Animals—120 albino Swiss mice, 54 inbred albino guinea pigs, 24 pure-bred New Zealand white rabbits, and 6 stock hybrid rabbits, 8 ground squirrels (*Citellus richardsonii* Sabine), and the chorio-allantoic membranes of chick embryos were tested for their susceptibility to the virus.

The animals were used in groups of from 2 to 4 when they were employed to determine species susceptibility to the virus, and in groups of from 2 to 6 when blind serial transfers of tissue were effected The blood picture of each animal was followed for several days before injection.

Virus of Infectious Feline Agranulocytosis—32 strains of virus have been employed. The original strain was derived from hepatic tissue and carried by rapid serial passage for 13 generations before being glycerinated (1) Subsequent to this first experience we have used in our studies strains isolated from liver, spleen, intestinal mucosa, feces urine respiratory washings, and blood

One strain of the virus of malignant panleucopenia (4 5)[2] and 3 strains isolated from the tissues of cats diagnosed clinically as feline enteritis were studied[2] and found to be indistinguishable pathologically clinically, and immunologically from the virus of infectious feline agranulocytosis

Other Materials Used for Inoculation—The viruses of hog cholera[2] fox encephalitis[2] B virus infection, herpes (HF strain) vesicular stomatitis equine encephalomyelitis (Western type), and lymphocytic choriomeningitis were employed in attempts to learn if any relationship to the causal agent of infectious agranulocytosis could be demonstrated

Preparation of Virus Suspensions for Inoculation—The tissues, which were used to provide the virus of feline agranulocytosis, were obtained from animals immediately after they died spontaneously or were killed by chloroform or ether at the height of the disease (as determined by the characteristic profound granulocytopenia) The suspensions containing each of the other viruses were prepared from glycerinated tissues. When liver spleen, brain or lung was utilized, the tissue was thoroughly

[2] We are indebted to F W Schofield and A W Bain of the Ontario Veterinary College, Guelph Ontario, and to W A. Hagan and W S Monlux of the New York State Veterinary College at Cornell University for glycerinated specimens of liver and spleen from cases diagnosed clinically as infectious feline enteritis, to J F Enders and W A Hammon of Harvard University Medical School for tissues and immune serum from cases diagnosed as malignant panleucopenia to R. E Shope for hog cholera virus contained in whole blood, and to R. G Green for fox encephalitis virus.

triturated with alundum[3] in Locke's solution to yield a 10 per cent suspension. Meat extract broth was substituted for Locke's solution when filtration experiments were contemplated. (Before trituration, tissues preserved in glycerin were first washed three times in Locke's solution to remove excess glycerin.) This suspension was centrifuged horizontally at 1500 R.P.M. for 30 minutes, and the supernatant fluid directly, or after filtration, was used undiluted or in decimal dilutions to inoculate animals.

The procedure outlined above necessarily had to be modified when intestinal mucosa, blood, or body secretions and excretions were employed. Unmodified whole blood and urine were used. On the other hand, nasopharyngeal washings, intestinal mucosa, and feces (10 per cent by weight) were suspended in meat extract broth in a flask containing glass beads and shaken for 60 minutes in a Camp six-flask shaking machine operated by a 1/8 horse power electric motor. The fluid suspension that resulted was centrifuged at 2500 R.P.M. for 1 hour, and the supernatant liquid withdrawn and filtered through a Berkefeld "V," "N," or "W" candle, or a Seitz E-K disc. The filtrate was used to inoculate animals.

Filtration.—Berkefeld candles of "V," "N," and "W" porosity, and Seitz E-K discs were employed. New candles were used for the earlier experiments and reused in later experiments. All candles were cleaned by the successive passage of saturated potassium permanganate, saturated oxalic acid, and water, and sterilized by autoclaving. Filtration was effected by negative air pressure obtained by attaching the filter to a water system. (A water bottle and mercury manometer were inserted between the filter and the water system.) The negative pressures in centimeters of mercury routinely employed for filtration were as follows: "V" candles, 10 cm.; "N" candles and Seitz E-K discs, 20 cm.; and "W" candles, 30 cm. *Serratia marcescens* was employed to test the impermeability of the filters to microbial agents. When a filter was found to be pervious to this bacterium, the results were not accepted in evidence of the filterability of the causative agent.

Preservation of Virus.—50 per cent glycerin buffered to a pH of 7.2 with phosphates, and freezing followed by dessication were the methods used for preservation of the virus.

Cultures.—In our earlier bacteriological studies, each virus suspension was used to inoculate a wide variety of media which included Douglas's broth, blood agar plates, Loeffler's media, Fletcher's media, media for growth of the pleuropneumonia group (7), deep meat tubes, and a modified Bordet-Gengou media for incubation under aerobic, anaerobic, and microaerophilic conditions. In later bacteriological studies only Douglas's broth and blood agar plates were inoculated with heart's blood, liver, and spleen, and incubated under aerobic and anaerobic conditions.

Pathological Examination.—Autopsies were performed on most of the animals whose death resulted spontaneously or from the purposeful use of an anesthetic. Tissues were fixed in Zenker's (5 per cent acetic acid) fluid, sectioned, and stained according to Giemsa's method or by means of hematoxylin and eosin.

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[3] Alundum, an electrically fused crystalline alumina, prepared by the Norton Company, Worcester, Massachusetts, was used because of its excellent "cutting" qualities.

RESULTS

Properties of the Feline Infective Agent

It became apparent early in these studies that the causative agent was not readily cultivable, and that it satisfied criteria which are accepted as characterizing a virus (1) Nevertheless the extreme contagiosity of the disease and the high incidence of immunity among stock adult cats led us to question if our positive transmission experiments employing filtrates for the inoculation of 24 animals (1), and the two positive transmission experiments employing filtrates for the inoculation of 4 animals, as reported by Hammon and Enders (4), constituted adequate evidence for the unequivocal filterability of the causative agent. Because of this reasonable doubt, we made every effort to recover a cultivable agent and thereby to eliminate bacteria, fungi, or pleuropneumonia like organisms.

Bacteriological Studies—The blood and visceral tissues were cultured by employing a wide variety of both common and special liquid and solid media (as described above), with incubation under aerobic, anaerobic, and microaerophilic conditions

It was found that extraneous contaminants were present only rarely and most irregularly Moreover, microscopic study of sections and smears prepared from all affected tissues have not disclosed recognizable bacteria or parasites, excepting in the tissues from the gastrointestinal and respiratory tracts.

It is apparent, therefore, that the results of the bacteriological studies supported other evidence which showed that the causative agent was a filterable agent.

Filterability—The agent readily traverses Berkefeld candles of "V," "N," and "W" grades, and Seitz E-K discs.

Typical feline agranulocytosis resulted (*a*) in 26 of 31 cats that were inoculated with Berkefeld "V" filtrates representing suspensions prepared in seven experiments from liver spleen, intestinal mucosa, feces lung or respiratory washings (*b*) in 7 of 10 animals inoculated with Berkefeld "N" filtrates representing suspensions prepared in two experiments from hepatic tissue, and (*c*) in 12 of 13 recipients of Berkefeld "W" filtrates prepared in four experiments from hepatic tissue.

It would appear from the foregoing results that the virus traverses Berkefeld "W" candles more readily than either Berkefeld "V" or "N" candles. A more likely explanation, however, is that the difference in results can be ascribed to the presence of immune animals among the recipients of the Berkefeld "V" and "N" filtrates. However, that may be, because of the ready filterability of the agent through Berkefeld "W" candles, the results of these filtration experiments have been interpreted to suggest that the virus is of relatively small size, probably 35μ or less Obviously, of course, ultrafiltration experiments in which graded collodion membranes of known average pore diameter

are employed must be carried out before acceptable information on this point will be forthcoming

Resistance to Glycerol and Drying—The infective agent of feline agranulocytosis is similar to other viruses in its resistance to glycerol and to desiccation when frozen

The virus, as contained in affected hepatic tissues, and kept in 50 per cent glycerol buffered at 7 2 for from 7 to 138 days, induced the characteristic disease in 4 of 11 cats employed in three experiments when the glycerinated tissue washed thrice in Locke's solution and prepared for inoculation was injected by the subcutaneous or intraperitoneal routes

The virus can be preserved by drying while in the frozen state

The virus, as contained in a 10 per cent suspension of hepatic tissue, was frozen at approximately −80°C in a mixture of cellusolve and solid carbon dioxide, and dried by high vacuum distillation from the frozen state The containers were then sealed under vacuum and stored at refrigerator temperature For use, the desiccated material was resuspended in sterile water, and 5 ml were inoculated intraperitoneally into each of 4 cats Two of these 4 animals developed typical feline agranulocytosis, one on the 8th and the other on the 9th day after injection

From this experiment it was concluded that the infectivity of the virus is not appreciably affected by desiccation *in vacuo*

Pathogenicity by Different Routes of Inoculation

Cats of all ages have been found to be susceptible to infection when virus is administered by a variety of routes of inoculation

Intraperitoneal Route—Passage of the virus in cats has ordinarily been effected by the intraperitoneal route This route seemed the most desirable because of the ease of administration and the assurance that all susceptible animals would contract the disease

After having remained well during the 12 to 92 day period of isolation, 34 groups of cats, containing 127 animals in all, were injected intraperitoneally with 5 ml of virus in suspension Of the 84 cats in the 21 groups that received unfiltered material, 49 had the typical disease, of 22 cats in 7 groups that received a Berkefeld "V" filtrate, 17 had the typical disease, of the 8 animals in 2 groups that received a Berkefeld "N" filtrate, 7 had the typical disease, and of the 13 cats in 4 groups that received Berkefeld "W" filtrate, 12 animals had the typical disease

The results that were obtained when virus was introduced by the intraperitoneal route give ample evidence that cats are highly susceptible to infection by this route On the other hand, the large number of cats that was recorded as not having developed the disease (35 or 84) can be ascribed to the

presence of immune animals among the recipients, and to the inclusion of only such cats as had unequivocal hematological evidence of the disease It should be noted that many of the cats found to be "refractory" to infection were injected with unfiltered suspensions of the virus Some of these cats were unselected stock adult cats and, therefore, undoubtedly had been naturally exposed to the infectious agent. As immature cats usually were employed in filtration experiments, on the other hand, a much greater percentage of the cats yielded unequivocal evidence of the disease.

In an effort to obtain a clue as to the natural mode of infection, virus was experimentally introduced by peripheral routes, which might indicate whether the virus was transferred in nature by droplet infection, contaminated food or water, or insect transmission Circumstantial evidence based on the extraordinary spontaneous communicability of the infection by cage, body, and room contact made it seem possible that any one or all of the aforementioned vehicles were active. The intranasal route was first investigated, for it seemed to be the most probable route for natural infection

Intranasal Route—The introduction of the filterable agent by this route resulted in typical disease

A single group of cats containing 6 animals was inoculated under light ether anesthesia by *dropping 0 1 ml of a tissue suspension containing virus into both nares.* Two of the 6 animals had characteristic hematological findings 9 and 10 days later, respectively

This single experiment made it clear that cats are susceptible to infection when virus is inoculated intranasally When virus is introduced by this route, however, it is most difficult to control its spread Accordingly, no further experiments employing the intranasal route were undertaken

Gastrointestinal Route—In a preliminary experiment, a 10 per cent fecal suspension was introduced by means of a rubber tube into the stomachs of 3 cats. Two of the 3 animals came down with the characteristic disease 4 and 17 days later

From the results of this first experiment, it appeared that cats are vulnerable to infection by virus contained within the gastrointestinal tract It was thought advisable, therefore, to carry out a second experiment to confirm the results of the first experiment and to test diluted fecal material for its infectivity

Samples of a fecal suspension representing 5 dilutions 10^1 10^3, 10^5 10^7, and 10^9, were introduced through a rubber tube into the stomachs of as many cats. Only the 2 animals that received the 10^1 and 10^3 dilutions contracted the disease.

The production in cats of typical feline agranulocytosis following the introduction by stomach tube of a fecal suspension was accepted as presumptive

for the 9th day, were inconsequential, however, for all 3 cats in this group were immune

These experiments show that virus is present in the blood stream, and that its infectivity is but little affected by heparin, sodium citrate, or dessication *in vacuo* when frozen As blood, therefore, constitutes a readily available source of virus, it was used in later experiments Moreover, the presence of virus in the blood stream throughout the preclinical period, when related to the demonstrated infectivity of virus inoculated by the cutaneous route, makes it apparent that the experimental criteria for possible arthropod transmission are satisfied

The presence of virus in the blood of animals with agranulocytosis suggested that the infectious agent is widely distributed in the tissues Accordingly, lung, spleen, and liver, as representative tissues, were tested for the presence of virus

In a single experiment, a Berkefeld "V" filtrate, prepared from pulmonary tissue, was used for the inoculation of 6 cats, 3 intraperitoneally and 3 subcutaneously All 6 had typical agranulocytosis from 6 to 8 days later, and 5 of the 6 died

Splenic tissues from 2 animals were used for the intraperitoneal injection of 2 groups containing 7 cats Of these 7 animals, 5 exhibited evidence of the typical disease

Hepatic tissues from 15 cats were used in 17 experiments for the inoculation of 80 animals Of these 80 cats, 44 had typical feline agranulocytosis

These results give ample evidence for the infectivity of pulmonary, splenic, and hepatic tissues Because of the ready availability and abundance of hepatic tissue, this material was used frequently and for a variety of purposes It is for these reasons that such a large number of animals was injected with hepatic-tissue suspensions, and therefore, are included in the present report The observations, which relate to changes in the dosages and methods of administration of the virus, are considered in the section that deals with "Pathogenicity by different routes of inoculation"

The question next arose as to whether virus is present in the eliminatory products of the body Accordingly, respiratory secretions, feces, and urine were investigated

Respiratory secretions were tested first because the high incidence and epizootic nature of the spontaneous disease and the ready communicability of the infection suggested that the malady is spread naturally by droplets of nasal spray However, the almost complete absence of nasal secretions in cats at the height of the illness, led us to add mucosal scrapings from the respiratory passages and turbinates to the respiratory washings

The material, which was tested for virus, was obtained from a single cat by mixing the meat extract broth washings from its nasal passages with the mucosal scrapings

from its nasal passages and turbinates. This mixture was triturated in broth and alundum, centrifuged, and filtered through a Berkefeld "V" candle to yield a filtrate which was used for the intraperitoneal inoculation of 3 cats Two of the 3 animals had the disease 7 days later

The results of this single experiment show that virus is present in the respiratory passages at the height of the disease, and suggest, therefore, that nasal secretions constitute one vehicle for the natural spread of the virus Since we showed in earlier experiments that the cat is susceptible to infection by the intranasal route, it becomes apparent that the respiratory passages alone can serve as the portal both of exit and entry for the virus.

In an attempt to demonstrate virus in the feces, five experiments were carried out. In three of these experiments, the fecal suspensions were introduced parenterally, and in the other two, the fecal suspensions were deposited within the stomach by means of a rubber tube.

Seven cats in groups of 2, 2, and 3 representing three experiments, were injected with Berkefeld "V" filtrates Of these animals, 6 had typical feline agranulocytosis on the 5th day after injection, 1 on the 6th day Four of the 7 died

In the fourth experiment, each of 3 cats was given 5 ml of a 10 per cent fecal suspension by stomach tube. Two of these animals developed the disease, 1 by the 4th day after inoculation and the other by the 7th day

Each of the 5 cats, which were employed in the fifth experiment, received 5 ml of one of the following dilutions of a Berkefeld "V" filtrate 10^{1}, 10^{3}, 10^{5} 10^{7}, or 10^{9} Of these 5 animals, the cats that were given the 10^{1} and 10^{3} dilutions came down with agranulocytosis.

These five experiments show that abundant virus is present in fecal suspensions. Indeed, it would have been surprising if the characteristic pathological changes in the intestinal mucosa were not associated with the presence of virus in the feces. Nevertheless, we realized, of course, that the virus present in the feces could have its origin in nasal secretions, which had been swallowed, and therefore, that the associated occurrence of pathological changes in the intestinal mucosa and of virus in feces merely suggested the intestinal mucosa as a source of virus. In an attempt to obtain further evidence on this problem, two additional experiments were undertaken to determine if virus is present in the mucosal cells of the ileum.

In the sixth experiment, the ileum of a single moribund cat was removed, washed carefully to remove all of the intestinal contents, and scraped superficially to remove the surface epithelium These scrapings were used to prepare a Berkefeld "V" filtrate, according to the technique outlined and the filtrate was injected intraperitoneally into each of 3 cats. All 3 had the typical disease 7 days later

Employing the same procedure for the seventh experiment, the mucosal lining of the ileum from another cat at the height of its disease was used to prepare a Berkefeld

"V" filtrate for the intraperitoneal injection of a group of 6 cats Two of the 6 animals were ill with the disease 5 and 7 days later

The results of the foregoing seven experiments show that virus is present in both the fecal content and mucosal lining of the intestine When this evidence is considered in relation to the results of preceding tests, in which it was shown that cats are readily susceptible to infection by virus introduced into the stomach by tube or through surgical exposure, it becomes apparent that the gastrointestinal route also can act as a natural portal both for the entry and exit of the virus

It seemed possible that virus might be excreted in the urine Three experiments were carried out to test the possibility

Urine was withdrawn suprapubicly by syringe from the bladder of animals killed at the height of their disease It was used without further treatment, in doses of from 2 to 5 ml intraperitoneally, for passage to groups of 3, 4, and 5 cats, respectively, in three experiments These experiments were carried out at intervals of several months

None of the first group of 3 cats showed evidence of disease, following the injection of urine, but these same animals contracted the disease 20 days later when injected with a Berkefeld filtrate of fecal suspension On the other hand, all of 4 animals in the second experiment and 3 of the 5 animals in the third experiment had agranulocytosis in from 5 to 11 days after a single injection of urine

The results of these three attempts to demonstrate virus in the urine of cats with feline agranulocytosis suggest that virus is present irregularly in the urine of cats at the height of the disease The demonstration of virus in two of three attempts, however, indicates that contamination by urine is another mechanism that must be considered in the natural spread of the disease

Susceptibility of Other Species

Hammon and Enders (4) have found the rabbit, guinea pig, ferret, and mouse to be insusceptible to infection by the feline virus, and Kikuth, Gönnert, and Schweickert (6) (employing an infective agent which appears to be identical with that described by Hammon and Enders, and by ourselves), found the dog (3 pups and their mother), *rhesus* monkey, rabbit, hamster, canary, hedgehog, and rat to be insusceptible The small number of animals that they employed and their failure to use "blind" serial passages, except for brain material in mice, however, led us to carry out further tests to determine the susceptibility of common laboratory animals

All animals were isolated for at least 7 days before blind serial transfers were carried out at 7 day intervals Blood counts and temperatures were taken bidaily The virus suspensions, which were used to initiate each passage transfer, came from a cat that had exhibited a typical agranulocytic blood picture and inclusion bodies, and had yielded virus of proven infectivity on transfer to 6 normal cats Because it was felt that the host cell virus relationship might manifest different affinities on transfer

in the tissues of species removed from the cat the susceptibility of 3 types of tissues and their capacity for carrying the feline virus were tested by from 3 to 6 blind serial transfers. The source materials for transfer were brain tissue, hepatic and splenic tissues, and intestinal washings inclusive of the mucosa The passage series employing brain tissue was initiated by injecting a 10 per cent hepatic-splenic suspension both intracerebrally and intraperitoneally and thereafter using brain tissue for passage. The hepatic splenic tissue suspensions and Berkefeld filtrates of intestinal washings were injected intraperitoneally

Rabbits —Four groups each containing 6 New Zealand white rabbits, were used Of the first group of 6 rabbits injected with virus of feline origin 4 were given a 10 per cent hepatic splenic suspension (2 intraperitoneally 5 ml 1 intracerebrally, 0 25 ml. and 1 both intracerebrally, 0.25 ml. and intraperitoneally 5 ml) Although none of these animals showed signs of infection each of these 3 blind' passages was carried for 3 more transfers in series. The intracerebral series was maintained by both intracerebral and intraperitoneal injection of brain tissue from the preceding generation the hepatic-splenic tissue series by the intraperitoneal injection of these same tissues from animals of the preceding generation, and the intestinal content series by the injection of Berkefeld filtrates prepared from the intestinal contents of the preceding generation. When none of these animals showed any evidence of infection, each passage series was terminated by making the final transfers to normal cats, which had been maintained under isolation The cats remained well as measured by the absence of (*a*) alterations in the blood picture, (*b*) clinical findings of disease, (*c*) temperature changes suggestive of disease and (*d*) histological changes in the ileum, thereby indicating that no virus was present in the tissues under test. Moreover, the susceptibility of these same cats was affirmed from 13 to 20 days later, when all contracted the typical disease on injection with a virus suspension of known infectivity

From this experiment we conclude that the rabbit is refractory to infection by the virus of feline agranulocytosis Not only were two attempts to pass virus directly from the cat to the rabbit unsuccessful, but also attempts to enhance the pathogenicity of the feline agent for the rabbit, by using 3 types of material (hepatic splenic tissues, brain, and intestinal washings) for "blind" serial passages through 4 successive transfers in rabbits, failed to yield evidence of activity Moreover, the failure of these materials to elicit any evidence of infection when returned to the feline host strongly indicates that the virus is incapable of setting up even an inapparent infection in the rabbit.

The next three experiments were carried out with only slight modifications to test the susceptibility of 3 other species of animal, the guinea pig the white mouse, and the ground squirrel (*Citellus richardsonii* Sabine)

Guinea Pigs —In the first test, each of 4 guinea pigs was injected intracerebrally (0 1 ml.) and intraperitoneally (2 ml) with a feline hepatic suspension of proven infectivity None of the animals showed any evidence for infection

The second test was designed and carried out in a fashion similar to the 'blind" serial passage transfer in rabbits which was described in the foregoing experiment. Three series of 4 passages each were effected, employing hepatic splenic tissues, brain

tissue, and intestinal washings, respectively, as vehicles for the infective agent in the attempted maintenance and carriage of the virus Twelve guinea pigs in 3 groups of 4 were used for each passage The hepatic-splenic tissue passages and the brain-tissue passages were initiated with a feline hepatic-splenic tissue suspension (2 ml intraperitoneally and 0 1 ml. intracerebrally), and the intestinal washings passage series with a Berkefeld "V" filtrate of feline intestinal washings (0 1 ml intranasally and 2 ml intraperitoneally) In the subsequent 3 passages, for which guinea pig materials were used, the anatomic sources of the inocula, dosages, and routes of inoculation remained the same Total leucocyte and differential counts were taken 1 to 3 days before injection and on the 3rd and 6th days after injection When the animals were killed on the 7th day after injection, the suspensions for passage were prepared by using tissues from all 4 of the animals used in each series After the fourth passage, the passage material from each series was used for the injection of 2 cats, and pieces of the lower ileum were removed for histologic study

No evidence for infection was observed in any of the 48 guinea pigs or 6 cats employed If any virus was transmitted after the initial passage of infective feline tissues, therefore, the amount was insufficient to become established in the foreign host.

Mice —Six successive transfers at 7 day intervals in each of 3 series were effected before the passage material, under test for its ability to carry virus, was returned to a feline host. For each passage, 3 groups of 6 mice of from 20 to 40 days of age were used as recipients for hepatic-splenic tissues, brain tissue, and intestinal washings, respectively The two passage series employing hepatic-splenic and brain tissues were initiated by using a feline suspension of hepatic-splenic tissue, and the passage series employing intestinal washings was initiated with a filtrate of feline intestinal washings For the brain tissue passage series, 0 1 ml was injected intracerebrally and 2 ml were injected intraperitoneally, and for the hepatic-splenic and intestinal washings series, 2 ml were injected intraperitoneally

A fourth and final passage series was carried out using groups of 6 mice for the passage of pulmonary tissue every 7th day for four successive transfers The first group of mice was injected with a virus suspension of feline origin, and successive groups of mice received a 10 per cent suspension of pulmonary tissue prepared from the lungs of all of the 6 mice, which had been used in the preceding passage Each mouse received 0 1 ml intranasally and 0 5 ml intraperitoneally

On completion of each passage series, a suspension of the tissue under test was used to inject cats

Of the 132 mice used in the four transfer series, the 18 mice used in preliminary experiments, and the 8 cats used to test the infectivity of mouse tissues representing the final passage, none gave any evidence, in the form of clinical, hematological, or histopathological findings, that the virus of feline agranulocytosis was infectious for mice [6]

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[6] These experiments inadvertently served another purpose, for they gave good evidence that the inbred Swiss albino strain of mouse, which is reared for use in this laboratory, was free from the viruses known to cause inapparent infections in laboratory mice, *viz*, infectious ectromelia (8), lymphocytic choriomeningitis (9), the virus pneumonias, as described by Dochez, Mills, and Mulliken (10), Gordon, Freeman, and Clampit (11), Horsfall and Hahn (12), and Nigg (13), and spontaneous encephalo-

Ground Squirrels—Four successive "blind" serial transfers in gophers were made. The 2 gophers that were employed in each transfer received virus by the intracerebral (0 1 ml.) intranasal (0 1 ml.), and intraperitoneal (1 ml.) routes. The 10 per cent feline splenic hepatic suspension previously described was utilized to initiate the series, and thereafter, hepatic and splenic tissues from the 2 gophers in each preceding passage were used to prepare suspensions for the inoculation of the next generation in the passage series When the final passage was terminated, representative portions of brain, liver, spleen, and intestinal contents were obtained from both gophers, and used to prepare suspensions. The suspension of intestinal contents was filtered through a Berkefeld 'V filter and the resultant filtrate was mixed with equal amounts of the unfiltered suspensions of brain, liver, and spleen. Each of 2 cats received 5 ml. of this mixture intraperitoneally

None of the gophers or cats employed in this experiment gave any evidence for infection by the feline agent

It is worthy of note that two attempts to establish the virus on the chorio-allantoic membrane of the developing chick failed to give any evidence locally for the pathogenicity of the virus, and, further, tissue suspensions prepared from these chorio-allantoic membranes failed to yield virus when they were returned to susceptible cats

It is apparent from the results of the experiments above that our attempts to produce infection in species other than the cat were uniformly unsuccessful. From 4 to 6 'blind" serial passages were made in rabbits mice, guinea pigs, and gophers, employing the intracerebral, intranasal, and intraperitoneal routes of injection for suspensions of brain, liver and spleen, and intestinal washings, respectively, for each species. The possibility that an inapparent infection was initiated and maintained by serial passages was ruled out when susceptible cats did not react to the injection of any of the tissues removed from the animals employed in the last passage transfer

SUMMARY

Thirty two strains of an infectious filterable agent, with properties that establish it as a virus, have been isolated from a malady of cats This disease can be readily recognized and differentiated from other feline diseases by blood studies, which make apparent the characteristic profound leucopenia and marked relative lymphocytosis in the absence of thrombopenia and appreciable anemia (Because the cytological pictures of the bone marrow and blood are essentially similar to those which characterize human agranulocytosis, we have

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myelitis of mice (Theiler's disease) (14) Because murine encephalomyelitis may not become manifest for 30 or more days, 3 mice, representing the fourth to sixth successive mouse passage in each series were permitted to live for from 30 to 40 days after the passages at 7 day intervals had been terminated None of these mice showed any evidence of abnormality either before or after their death.

named the disease under study "infectious feline agranulocytosis") The cytological reaction to the presence of the virus is further characterized by proliferation of the reticuloendothelial cells of the lymph nodes and spleen, and by the formation of intranuclear inclusion bodies in the cells of the gastro-intestinal mucosa, lymph nodes, and bronchial mucosa

The etiological agent, the virus of infectious feline agranulocytosis, is pathogenic for cats when given by the oral, intragastric, cutaneous, subcutaneous, intraperitoneal, intravenous, and intranasal routes, it can be recovered at the height of the disease from the blood, spleen, liver, lung, intestinal mucosa, nasal secretions, nasal mucosa and turbinates, feces, and urine The virus appears to be limited in its pathogenicity to the feline species We found that a variety of animals, as represented by albino Swiss mice, guinea pigs, domestic rabbits, and ground squirrels (*Citellus richardsonii* Sabine), failed entirely to react to the injection of massive doses of virus Repeated attempts at infection of these animals regularly failed when the intranasal, intraperitoneal, subcutaneous, and intramuscular routes of inoculation were employed for single doses The same was true when from four to six transfers in "blind" serial tissue passages were made Moreover, attempts to propagate the virus on the chorio-allantoic membrane of the developing chick were unsuccessful

The significance of the facts is discussed in the paper that follows

THE VIRUS OF INFECTIOUS FELINE AGRANULOCYTOSIS*

II Immunological Relation to Other Viruses

By J S LAWRENCE, M.D., J T SYVERTON, M.D, R. J ACKART, M.D,‡ W S. ADAMS,‡ D M. ERVIN‡ A. L. HASKINS, Jr.,‡ R. H SAUNDERS, Jr.,‡ M B STRINGFELLOW M.D.,‡ and R. M WETRICH‡

(From the Departments of Medicine and Bacteriology The University of Rochester School of Medicine and Dentistry, Rochester, New York)

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Infectious feline agranulocytosis (1–3) is a disease entity in which the tissues, fluids, and excretory products yield a characteristic virus that is highly infectious by a wide variety of routes for members of the cat family, as the experiments in the preceding paper have shown. The immunological relation of this newly described virus to other viruses is considered in the present paper

Tests for Active Immunity

It was necessary to learn first whether cats that recover from either the spontaneous or experimental infection (irrespective of the route of inoculation) are solidly resistant to reinfection by massive doses of virus, as measured by the absence of the accepted clinical, hematological, or pathological evidence of disease In an experiment to decide the point, cats known to have had the *typical disease in from a few days to many months previously* were tested by the parenteral injection of virus for their capacity to resist reinfection.

Thirteen cats, which were known to have had either the spontaneously or the experimentally induced disease in from 4 to 288 days previously, were tested for immunity by the intraperitoneal injection of a single massive dose of virus, consisting of from 3 to 5 ml. of hepatic tissue suspension.

All of the animals proved refractory to reinfection

It is apparent from the results of this experiment that recovery from infectious feline agranulocytosis is followed by complete resistance to reinfection. Furthermore, this evidence was confirmed repeatedly under natural conditions, for the disease has never been observed to recur in cats returned to the animal house after recovery Susceptible cats, on the other hand, regularly develop the disease spontaneously shortly after admission to the same quarters

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‡ Student Fellow in Medicine

Susceptibility of Normal and Immune Cats to Other Agents

Experiments were undertaken in an attempt to demonstrate a relationship between the virus of infectious feline agranulocytosis and other agents These experiments fall into three groups Group A, consisting of three experiments, employed normal cats in two experiments and immune cats in the third The animals in these experiments received a test dose of an agent conceivably related to the virus of feline agranulocytosis that was followed 3 weeks later by a challenge dose of the feline agent

As the peripheral blood picture in hog cholera (15) is suggestive of that of feline agranulocytosis (the pathological changes in the various organs were not reported), hog cholera virus was employed in the first experiment to determine whether clinical symptoms and changes in the blood and bone marrow would result in cats infected by this virus

5 ml of whole blood containing hog cholera virus were inoculated intraperitoneally into 5 of 6 cats that had been in isolation for from 25 to 71 days None of these animals showed any clinical or hematological evidence of infection However, when these 6 animals were injected with a challenge dose of feline agranulocytosis virus 21 days later, the control and 3 of the 5 test animals developed typical agranulocytosis

From the results of this first experiment, it was concluded that the cat is refractory to infection by hog cholera virus, and that there is no apparent relationship between the viruses of hog cholera and feline agranulocytosis

Fox encephalitis virus was used for the next two experiments The only reasons for selecting this virus were the slightly suggestive resemblances between the clinical and pathological pictures of the two diseases, and because in so far as we know, the blood picture of fox encephalitis has not been studied Accordingly, Experiments 2 and 3 were planned to test normal cats and cats immune to infection by the virus of feline agranulocytosis for susceptibility to infection by the virus of fox encephalitis

Each of the 4 test animals in each experiment was inoculated under light ether anesthesia with a 2 per cent virus suspension, 0 8 ml intracisternally, and 4 ml intraperitoneally As controls, single animals were maintained under identical conditions but were not injected

None of these animals yielded any evidence to suggest that fox encephalitis virus was pathogenic for the cat, or that it had any effect on the peripheral blood picture of this animal Moreover, these results were substantiated further when a challenge dose of virus was given to each animal, for none of the immune animals showed any evidence of feline agranulocytosis infection, whereas 4 of the 4 normal cats developed the clinical disease.

These two experiments convince us that cats are insusceptible to infection by the virus of fox encephalitis, that this virus does not alter the peripheral blood

picture, and that there is no apparent immunologic relationship between the two viruses.

The experiments in group B were designed to learn whether the cat is susceptible to infection by any one of the five viruses equine encephalomyelitis (Western type), vesicular stomatitis (Indiana type), lymphocytic choriomeningitis (W S strain), B *virus infection*, and *herpes* (*HF strain*)

For each experiment a single virus in 10 per cent suspension was employed for the inoculation of 2 cats, 0 25 ml. intracerebrally and 1 ml intraperitoneally

Of the 10 cats injected, only a single animal, injected with vesicular stomatitis virus, developed signs of disease as shown by a bilateral paralysis involving both hind limbs. Attempts to recover the virus of lymphocytic choriomeningitis virus from the spleens of cats that had received the virus intraperitoneally and intracerebrally were unsuccessful

The results of this second group of experiments make it apparent that of the viruses, when tested by us,—the Western type of equine encephalomyelitis, vesicular stomatitis, lymphocytic choriomeningitis, B virus infection, or herpes —none is pathogenic for the cat.

Studies on the Identity of the Causal Agents of Infectious Feline Agranulocytosis, Malignant Panleucopenia of Cats, and Infectious Feline Enteritis

When the first paper by Hammon and Enders was published (4), it was obvious that they were working with a disease identical or closely related to the disease which we had described (1) It seemed desirable, therefore, to make comparative studies of the infective agents of these two feline maladies (16) Drs Enders and Hammon kindly supplied immune serum and glycerinated tissues, consisting of the spleen, lymph node, and bone marrow from one animal, and splenic tissue from another These materials were used in two experiments

The first experiment was designed to show whether tissues from cases diagnosed as malignant panleucopenia contain an agent that would give rise to a disease clinically and hematologically identical with feline agranulocytosis

The supernatant fluid of a 10 per cent suspension, prepared from representative portions of the glycerinated tissues supplied by Drs. Enders and Hammon, was used in 4 ml. amounts for the inoculation of 9 cats. Of these 9 animals, 3 were normal and 6 had recovered from feline agranulocytosis.

All 3 of the normal animals developed typical feline agranulocytosis in from 6 to 8 days after injection whereas the 6 agranulocytosis-immune animals showed no evidence to suggest either illness or an altered blood picture.

This first experiment makes it evident that tissues removed from cats with malignant panleucopenia contain an infectious agent that gives rise to a disease

with clinical and hematological features indistinguishable from those of feline agranulocytosis Added evidence to support the identity of the two agents is the refractoriness of agranulocytosis-immune cats to infection by the agent of panleucopenia

Further evidence to support these findings was sought in the next experiment in which panleucopenia-immune serum was tested for its protective effect against infection by agranulocytosis virus

Each of 3 normal cats was injected intraperitoneally with 5 ml of the test serum, and within a few minutes 4 ml of a suspension of feline agranulocytosis virus was injected subcutaneously Each of 11 cats injected intraperitoneally with an identical amount of the same preparation of virus served as a control (Because we wanted cats with the disease for other purposes we made the control group unusually large)

None of the test animals contracted the disease, whereas the typical disease developed in 10 of the 11 animals serving as a control

It was concluded that the results of these two experiments establish the identity of the two viruses

Infectious feline enteritis is a second disease that somewhat resembles feline agranulocytosis in its clinical picture, but published descriptions characterize it as a severe enteritis Moreover, the blood picture in feline enteritis has not been described Accordingly, it seemed desirable to make comparative studies of this disease and feline agranulocytosis As a strain of the virus of feline enteritis was not available, we sought tissues from cats with an illness diagnosed as feline enteritis by qualified veterinarians Of three requests made to leading schools of veterinary medicine, two yielded tissues for study The procedure followed and the results obtained were identical with those described in the study of the agent of panleucopenia

It was found that both samples of tissue yielded an infectious agent that is identical with the virus of feline agranulocytosis We feel that these studies are inconclusive, however, because the peripheral blood picture of the source animals was not studied It is impossible to say positively, therefore, that the cases, which were diagnosed clinically as feline enteritis, were not feline agranulocytosis

DISCUSSION

The data presented in the present paper and in that preceding it show the virus of infectious feline agranulocytosis to be the causal agent of a highly infectious disease of cats To promote consideration of the significance of our observations, some of the results of these studies will be briefly stated

The feline malady is characterized by an extreme granulocytopenia, marked relative lymphocytosis, less pronounced leucopenia, hypoplasia with the absence of differentiation of the myeloid cells of the bone marrow, prolifera-

tion of the reticuloendothelial cells of the lymph nodes and spleen, and intranuclear inclusion bodies in the cells of the gastrointestinal mucosa, lymph nodes, and bronchial mucosa. The high infectivity of the virus for the cat is manifest when it is inoculated by the oral, intragastric, intranasal, cutaneous, subcutaneous, intraperitoneal, and intravenous routes, but its pathogenicity is limited to feline hosts. The virus is widely distributed in the host's tissues and fluids, for it is readily recovered at the height of disease from the blood, liver, spleen, lungs, nasal mucosa and turbinates, nasal secretions, intestinal mucosa, feces, and urine. All strains of the virus that have been tested are immunologically identical.

The complete avirulence of the virus of feline agranulocytosis for any species other than the cat seems to distinguish this agent from the viruses whose pathogenicity for other species is well established. Our inability to infect white mice, guinea pigs, rabbits, ground squirrels (*Citellus richardsonii* Sabine), and the chorio-allantoic membrane of the developing chick confirms and extends susceptibility tests with animal species other than the cat, as reported by Hammon and Enders (4), and by Kikuth, *et al.* (6). On the basis of species susceptibility, therefore, the virus appears to be distinct from the etiological agents of lymphocytic choriomeningitis, influenza, Rift Valley fever, louping ill, canine distemper, fox encephalitis, mouse encephalomyelitis (Theiler), the pneumonia carried by normal mice, as described by Horsfall and Hahn (12), infectious ectromelia, vesicular stomatitis, equine encephalomyelitis, St. Louis encephalitis, the pox group, and the meningopneumonitis-psittacosis-lymphogranuloma venereum group. These viruses are further distinguished from the virus of infectious feline agranulocytosis by distinctive differences in the pathological findings that result from infection. Moreover, further evidence for our belief in the singleness of identity of the virus of feline agranulocytosis was our inability to establish clinical infections in the highly susceptible cat by the inoculation of viruses that readily infect a variety of laboratory animals. Thus, we found the cat to be clinically refractory to infection by the viruses of hog cholera, lymphocytic choriomeningitis, fox encephalitis, vesicular stomatitis, the Western type of equine encephalomyelitis, herpes, and B virus infection.

Because the disease is extremely contagious, Hammon and Enders (4), and Kikuth, Gönnert, and Schweickert (6), were unable to prevent animals from contracting the disease spontaneously (except in two experiments reported by Hammon and Enders[1]). We have had similar results when cats were introduced into our usual animal quarters. In the light of these ex-

[1] Hammon and Enders (4) reported two experiments in which they used two widely separated farms to maintain 7 cats in 2 groups without evidence of disease for 21 and 27 days, respectively. Following the period of isolation, 3 of the 4 cats, which were inoculated with filtrates, gave unequivocal evidence of the disease.

periences, therefore, it is worthwhile emphasizing that all of the cats employed in the present experiments were kept under rigid isolation for from 12 to 92 days before being used During this time each cat was observed daily for clinical signs of disease, and its hematological status was followed by bidaily total cell and differential studies of the white blood cells Thus, we were enabled to eliminate immediately any group in which a single member showed overt signs of illness Our results, therefore, are based on the use of cats that were kept in isolation for a period that greatly exceeded the incubation period of the disease Moreover, each group of animals under study was controlled further by the inclusion of 1 or more normal cats, which were not injected

In order to understand the epizoology of any infectious disease, it is important to know the routes whereby the etiological agent can enter and leave the body of its host In the present study, our experimental data suggest the gastrointestinal and respiratory routes as natural portals for the virus to infect the cat, and the cutaneous route as a possibility The proven susceptibility of the cat to virus introduced intranasally and the extraordinary communicability of the disease make it apparent that the disease can be transmitted naturally by the respiratory route On the other hand, the ready susceptibility of the cat to virus introduced by mouth or stomach tube, the pathological changes in the epithelial cells of the intestinal mucosa, and the massive amounts of virus excreted in the feces suggest contaminated food as a natural source of infection Of significance too, perhaps, were repeated observations that administration of the virus by the oral or intragastric route resulted in an incubation period as short, or shorter, than by any other route Moreover, the presence of virus in the urine of infected cats increases the probability that contaminated food acts to spread the disease Most likely both are natural routes Although the cutaneous route is a possibility, as has been shown by the demonstrated presence of virus in the blood stream and the infectivity of virus inoculated by the cutaneous route, it seems unnecessary to assume that the virus is spread by a biting arthropod Our knowledge can be summarized, therefore, by stating that the natural vehicle for the spread of the disease could be nasal droplets, contaminated food, or contaminated arthropods

As with each new infectious agent, it is difficult to learn whether the disease under investigation, or its causative agent, has been described previously Such is the case with the virus of infectious feline agranulocytosis It early became apparent from a survey of the literature related to epizootic diseases of cats, however, that a feline disease with the distinctively characteristic hematological and pathological findings of feline agranulocytosis had not been reported Soon after our note (1), Hammon and Enders described a disease (4) (which they named malignant panleucopenia in a later publication (5)) that was proven by immunological studies (16) to be caused by a virus identical with the virus under investigation Moreover, Kikuth, Gonnert, and Schweickert (6) have described what appears to be the same disease in

Germany, and which they named "infectious aleucocytosis of cats" Thus, it appears that a single disease has been given three names as the result of studies carried out in three widely separated laboratories Moreover, the problem was recently complicated further by the recovery of 2 strains of this same virus from cases diagnosed as feline enteritis by two highly competent veterinarians. These last results suggest two possibilities either the virus under investigation is identical with that of feline enteritis, or the clinical designation "feline enteritis" may be employed loosely to cover a variety of feline maladies, which have in common involvement of the gastrointestinal tract If the first possibility be right, then it is remarkable that the extensive blood changes and the formation of inclusion bodies, which are known to occur regularly in feline agranulocytosis, had not been discovered by earlier workers (17) On the other hand, it is not surprising that a variety of feline illnesses should be caused by filterable agents, or that these illnesses should be accompanied by signs and symptoms related to the gastrointestinal tract, for it is well known that human maladies of virus etiology without inappetance, nausea, vomiting, or diarrhea are rare indeed. Therefore, it becomes apparent that the mere presence of signs or symptoms related to the gastrointestinal tract are not adequate evidence for a specific diagnosis. It remains a question, therefore, as to how this clinical entity should be designated The infectious nature of the disease, the strict limitation of its host range to cats, and a cytological picture of the bone marrow and blood that is indistinguishable from that of human agranulocytosis, suggested the name "infectious feline agranulocytosis." Certainly if agranulocytosis is a satisfactory name for the human syndrome, then the feline disease should be so designated Malignant panleucopenia, on the other hand, is not as descriptive and is misleading, for it implies an essentially fatal disease in which all the white cells of the blood are involved equally That the marked leucopenia and neutropenia are accompanied by a relative lymphocytosis was shown previously (3), and that the disease is not uniformly fatal was established in the same investigation Moreover, these earlier findings are substantiated by the results of the present study in which fully as many of the cats tested (over 400) were shown to have an acquired immunity as to be susceptible. Thus, it would seem that "malignant panleucopenia" is ill suited as a name for this disease.

The status of the name 'feline enteritis," on the other hand, is not readily disposed of, for feline enteritis is a disease that is accepted by veterinarians as a clinical entity caused by a filterable virus Moreover, two competent diagnosticians found the disease under investigation to be indistinguishable from illnesses that they considered clinically to be feline enteritis. When it is realized, however, that the term feline enteritis is often used in veterinarian circles to cover any infectious malady with clinical signs pointing to the gastrointestinal tract, and that the hematological aspects of feline enteritis have never been investigated, it is difficult to know whether "feline enteritis" refers

to a single specific disease which has not been studied hematologically, or whether the term is used as a clinical designation for a variety of maladies that are included under a single name It seems advisable, therefore, to retain and to perpetuate the name "infectious feline agranulocytosis," and to encourage veterinarians to use blood studies for the separation of the entity from other feline maladies

SUMMARY

The infection of cats by the virus of infectious feline agranulocytosis is followed by the production of specific neutralizing and protective antibodies, and recovery from the disease is associated with the development of solid immunity to reinfection From the evidence presented it is obvious that the virus is not related to the viruses of hog cholera, lymphocytic choriomeningitis, fox encephalitis, vesicular stomatitis, the Western type of equine encephalomyelitis, herpes, and B virus infection

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STUDIES CONCERNING THE SITE OF RENIN FORMATION IN THE KIDNEY

IV THE RENIN CONTENT OF THE MAMMALIAN KIDNEY FOLLOWING SPECIFIC NECROSIS OF PROXIMAL CONVOLUTED TUBULAR EPITHELIUM*

BY MEYER FRIEDMAN, M.D., AND ALEX KAPLAN, PH.D

(From the Harold Brunn Institute for Cardiovascular Research Mount Zion Hospital, San Francisco)

PLATES 3 AND 4

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In the first two studies (1, 2) of the present series, it was observed that whereas the kidney of fresh water fish contained renin, the kidney of marine fish did not, regardless of whether the kidney of the latter type of fish contained glomeruli or not. In our third study (3), it was found that the mesonephros and metanephros of the developing hog fetus contained renin despite the fact that neither type of kidney possessed specialized juxtaglomerular cells as described by Goormaghtigh (4) Furthermore, in this last study, it was found that the renin content of either type of embryonic kidney was dependent not upon its arteriologlomerular component but upon the activity and structural integrity of its convoluted tubular mass

Nevertheless, the relationship of intact tubular mass and function to the formation of renin in the adult mammalian kidney has not yet been ascertained. The dependence of both the glomerular and tubular components of the kidney upon a common blood supply makes the eradication of one component alone, quite difficult However, it was reported by Underhill, Wells, and Goldschmidt (5, 6) and later confirmed by Potter and Bell (7), that the subcutaneous administration of tartrate to a rabbit effects a differential necrosis of its renal convoluted tubules, affecting other portions of the nephron This method then, offers us the opportunity of comparing the renin content of a kidney possessing normal arteriologlomerular and tubular components with one possessing a normal arteriologlomerular component but a severly damaged proximal convoluted tubular mass A significant decrease in the renin content of the latter type of kidney would indicate that this portion of the mammalian kidney was capable of forming or storing renin. The results of such a study are reported in this communication

Methods

Thirty two rabbits were injected subcutaneously with a neutral solution of tartaric acid as described by Underhill *et al* (5) The amount injected, varied from 0 765

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TABLE I

The Pressor Substance (Renin) Content of the Kidney of Normal and Tartrate Injected Rabbits

Rabbit kidney	Recipient dog	Duration of life after tartrate injection	Extract injected (dry kidney powder)	Mean arterial pressure: Before injection	Mean arterial pressure: After injection of rabbit kidney extract	Pressor effect (rise per gm of dry kidney powder)
(a) Kidney of Normal Rabbit						
		hrs	*gm*	*mm Hg*	*mm Hg*	*mm Hg*
(1) R-40	21	—	1 00	113	165	52 0
(2) R-41	93	—	1 00	126	151	25 0
(3) R-42	82	—	1 00	134	156	22 0
(4) R-43	92	—	1 00	127	185	58 0
(5) R-49	79	—	1 00	129	148	19 0
(6) R-61	90	—	1 00	139	170	31 0
(7) R-89*a*	71	—	1 00	139	158	19 0
R-89*b*	64	—	1 00	152	184	32 0
(8) R-90	71	—	1 00	130	159	29 0
Average						32 0
(b) Kidney of Rabbit Injected with Tartrate (without Tubular Necrosis)						
(1) R-19*	87	48	1 00	109	161	52 0
(2) R-24*	21	96	1 00	97	151	54 0
(3) R-26	92	96	1 00	133	190	57 0
(4) R-27	92	144	1 00	140	156	16 0
(5) R-28	21	144	1 00	115	156	41 0
(6) R-29	85	144	1 00	121	147	26 0
(7) R-30	84	144	1 00	128	170	42 0
(8) R-32	83	96	1 00	110	122	12 0
(9) R-33	21	96	1 00	124	166	42 0
(10) R-35	21	96	1 00	117	123	6 0
(11) R-36	21	96	1 00	109	140	31 0
(12) R-37	83	96	1 00	138	178	40 0
(13) R-38*	86	96	1 00	140	154	14 0
(14) R-39	83	96	1 00	110	139	29 0
(15) R-50	88	96	1 00	119	153	34 0
(16) R-51*	92	96	2 00	130	194	32 0
(17) R-59	80	96	1 00	146	164	18 0
Average						32 0
(c) Kidney of Rabbit Injected with Tartrate (with Moderate Tubular Necrosis)						
(1) R-21	92	48	1 00	106	112	14 0
(2) R-23	86	72	1 00	153	170	17 0
(3) R-45	83*a*	48	1 00	130	132	2 0
(4) R-46	88	48	2 36	102	130	12 0
(5) R-47	95	48	1 00	119	132	13 0
(6) R-48	95	48	2 49	122	151	12 0
(7) R-52	78	96	1 00	155	168	13 0
Average						11 8

* Kidney showed marked tubular edema.

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TABLE I—*Concluded*

Rabbit kidney	Recipient dog	Duration of life after tartrate injection	Extract injected (dry kidney powder)	Mean arterial pressure		Pressor effect (rise per gm. of dry kidney powder)
				Before injection	After injection of rabbit kidney extract	
(d) Kidney of Rabbit Injected with Tartrate (with Severe Tubular Necrosis)						
		hrs	*gm*	*mm Hg*	*mm Hg*	*mm. Hg*
(1) R-81	64	48	2 00	156	156	0 0
(2) R-82	97	48	2 00	148	160	6 0
(3) R-83	70	48	2 00	140	152	6 0
(4) R-84	71	48	2 00	135	135	0 0
(5) R-85	71	48	2 00	148	148	0 0
(6) R-86	64	48	2 00	157	157	6 0
(7) R-87	72	72	2 00	122	122	0 0
(8) R-88	71	48	2 00	153	153	0 0
Average						2 3

to 1 75 gm of sodium tartrate per kilo of body weight It was found that when the maximal amount was given, the incidence of tubular necrosis increased markedly All rabbits were watched closely following injection and it was observed that those having severe tubular damage, became listless and obviously ill as early as 48 hours after the administration of the tartrate, whereas those rabbits showing little or no tubular damage on later histological examination appeared well even after 6 days, at which time, many were sacrificed It is important to emphasize that all kidneys were removed and extracted immediately after the rabbits were killed

After blocks for sections were obtained the kidneys of the rabbits were minced, ground, defatted, and desiccated as previously described (1) The dry powder resulting from such procedures, was extracted then for renin according to the method of Helmer and Page (8) The process was carried to the "Fraction B" stage (8) The physiological assay of the pressor substance (renin) content of each kidney extract was carried out on normal, anesthetized (pentobarbital sodium) dogs according to methods previously described (1) Because the kidneys of rabbits receiving tartrate were frequently edematous on removal the increase in blood pressure following the introduction of any kidney extract was expressed as mm. Hg rise per gram of dry kidney powder

For control purposes the kidneys of ten normal rabbits were treated and assayed later for renin as described above so that during any extraction and assay of a batch of kidneys obtained from rabbits injected with tartrate a normal kidney also was extracted and assayed Two of the control kidney extracts have not been included in Table I *a* because it was discovered that the test dog used was unduly sensitive to the pressor effect of injected renin

Description of Rabbit Kidneys Following Tartrate Injection

The kidney of a rabbit injected with tartrate was found to appear normal, edematous, or severly necrotic, depending upon the duration of the experiment

and the amount of tartrate injected. Almost every kidney obtained from rabbits within 24 hours following tartrate administration, exhibited gross enlargement which on histological examination was found to be due to edema of the proximal convoluted tubules (Figs. 1 and 2). This portion of the nephron was identified by its characteristic ciliated epithelium. If the amount of tartrate injected were under 1 gm. per kilo, the initial edema tended usually to subside so that the kidney of a rabbit allowed to live 4 to 6 days after tartrate injection, showed little evidence of edema or of tubular necrosis.

However, the kidney of a rabbit receiving over 1 gm. of tartrate per kilo, frequently developed a severe and widespread tubular necrosis (Figs. 3 and 4), which was limited for the most part to the epithelium of the proximal convoluted tubules. The glomerulus, even in the most damaged kidneys appeared quite normal, as has been previously reported, (5–7). Red blood cells could be seen in the capillaries, there was no hyperplasia of the capillary endothelium, no accumulation of leucocytes in the glomerular tuft, and the patency of the glomerular capillary was confirmed by the detection of carbon particles in its capillary loops after postmortem injection of India ink into the renal artery.

The Renin Content of Rabbit Kidneys Following Tartrate Injection

Physiological assay of various kidney extracts made it clear that there was no essential difference between the content of pressor substance (renin) of a kidney from normal rabbit and that of a kidney obtained from a rabbit injected with tartrate unless the latter showed actual necrosis of the proximal convoluted tubular epithelium.

Thus, as Table I*a*, *b* indicates, the average pressor effect (32.0 mm. Hg per gm. of dry kidney powder) obtained from eight different extracts of kidneys, (obtained from a corresponding number of normal animals), was identical with that obtained from 17 different extracts of kidneys of rabbits injected with tartrate without a resulting necrosis of proximal convoluted tubular epithelium. In other words, the administration of tartrate *per se*, to rabbits, did not alter the renin content of their kidneys even in those cases in which marked tubular edema had occurred.

However, it was observed, as shown in Table I*c*, *d*, that a kidney sustaining tubular necrosis following tartrate injection also showed a diminution in pressor substance (renin) roughly comparable to the extent and degree of the necrosis. Thus, in seven extracts of kidneys whose proximal convoluted tubular epithelium was only partially destroyed, the average pressor effect was 11.8 mm. Hg per gm. of dry kidney powder. In eight extracts of kidneys whose proximal convoluted tubular epithelium was widely destroyed, the average pressor effect was 2.3 mm. Hg per gm. of dry kidney powder. Thus, the normal kidney was found to contain approximately 14 times as much pressor substance (renin) as the kidney whose proximal convoluted tubular epithelium was necrotized severely by tartrate injection. It should be emphasized again that in both

normal and tartrate damaged kidneys, the arteriologlomerular component, including the specialized "Goormaghtigh cells" invariably appeared normal

DISCUSSION

In the first two studies (1, 2) of this series, the apparent absence of renin in the glomerular or aglomerular kidney of the marine fish as compared with its abundant presence in the kidney of fresh water fish, furnished the first, indirect, indications that the site of renin formation was in the tubular component of the kidney For it is known (9) that a difference exists between the renal tubular components of the two types of fish

In the third study (3) of this series, it became evident that the site of renin formation in the kidney (mesonephros or metanephros) of the hog fetus was tubular and had no detectable relationship with other portions of the kidney The complete absence of specialized juxtaglomerular cells in either the mesonephros or metanephros further strengthened this view

In the present and final study of this series, the almost complete disappearance of the pressor substance (renin) from an adult mammalian kidney whose proximal convoluted tubular epithelium had been destroyed, leads to the inescapable conclusion that in the adult mammalian kidney, the epithelium of the proximal convoluted tubules is concerned in the formation or storage of renin.

CONCLUSIONS

1 The administration of tartrate to adult rabbits was found to produce in some of them, a profound and widespread necrosis of the proximal convoluted tubular epithelium without affecting the other portions of the nephrons

2 The markedly damaged kidneys were found to be almost completely devoid of pressor substance (renin), indicating that in the mammalian kidney, the epithelium of the proximal convoluted tubules is concerned in the formation or storage of renin.

The authors wish to express their thanks to Eleanor Williams, Eleanor Kruger, and Helen Mendelson for their technical assistance.

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EXPLANATION OF PLATES

PLATE 3

FIG 1 Kidney of rabbit which had received 1 gm of tartrate per kilo 12 hours before Note the extreme hydropic degeneration of the convoluted tubular epithelium and the normal appearance of the glomerulus Between the edematous proximal convoluted tubules, normal appearing distal convoluted tubules can be seen Hematoxylin and eosin × 100

FIG 2 A section of same kidney as shown in Fig 1 under greater magnification Note the intense hydropic degeneration of proximal convoluted tubules and the normal appearance of the distal convoluted tubules immediately adjacent to the glomerulus Hematoxylin and eosin × 100

(Friedman and Kaplan Site of renin formation in kidney IV)

PLATE 4

FIG 3 Kidney of rabbit which had received 1 5 gm of tartrate per kilo, 48 hours before Observe the complete transformation of the epithelium of the proximal convoluted tubules into hyaline necrotic masses At the top of the section, intact epithelium of distal convoluted tubules may be seen, also normal appearing glomerulus Hematoxylin and eosin × 100

FIG 4 A section of same kidney as shown in Fig 3, under greater magnification Intact glomerulus can be seen, surrounded by necrotic epithelium of proximal convoluted tubules There are several distal convoluted tubules present which have not become necrotic Hematoxylin and eosin × 100

EPIDEMIC KERATOCONJUNCTIVITIS*‡

I Isolation and Identification of a Filterable Virus§

By MURRAY SANDERS M.D., and R C. ALEXANDER

(From the Departments of Ophthalmology and Bacteriology, College of Physicians and Surgeons Columbia University, and the Institute of Ophthalmology, Presbyterian Hospital, New York)

Plates 5 to 7

(Received for publication, September 25, 1942)

The problem of epidemic keratoconjunctivitis has gained prominence in recent years, because of the heavy incidence of this disease in shipyards and other places where industries are concentrated Aside from the fact that a menace to defense endeavors may be important during time of war, any disease which can temporarily incapacitate thousands of individuals, which is epidemic in character, and may produce some permanent reduction of vision, must be considered an important danger to public health There is no doubt that this infection, hitherto of unknown etiology, is a disease *sui generis* and can be differentiated from other conditions affecting the cornea and the con junctiva. For a complete clinical picture and differential diagnosis, the report of Hogan and Crawford (1) describing the 1941 epidemic on the Cali fornia coast, and of Rieke (2) describing 600 cases in Oregon, may be consulted In the Institute of Ophthalmology, the criteria which have been considered characteristic of epidemic keratoconjunctivitis are as follows an acute follicular conjunctivitis with a scanty exudate, preauricular lymph node enlargement and tenderness, negative bacteriology, mononuclear cellular exudate, and a punctate keratitis, with lesions of varying size. This corneal involvement, occurring 1 week to 10 days after the onset of the disease, is found in 30 to 80 per cent of the cases.

In a previous report (3), preliminary findings were given concerning the recovery of an infectious agent from two patients suffering with epidemic keratoconjunctivitis Evidence was presented suggesting that the agent is a virus, and its neutralization by serum from patients convalescent from the

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‡ This investigation was carried on in informal collaboration with the Commission on Neurotropic Virus Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the United States Army

§ The authors wish to extend their thanks to Dr Phillips Thygeson for his kind cooperation in this investigation

First Isolation
Epidemic Keratoconjunctivitis

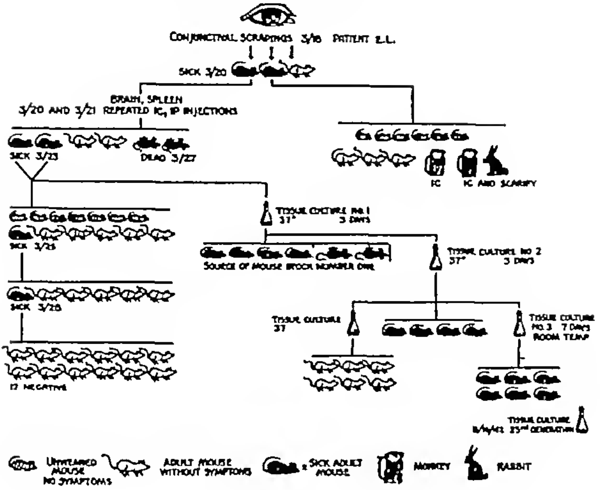

DIAGRAM 1

Second Isolation
Epidemic Keratoconjunctivitis

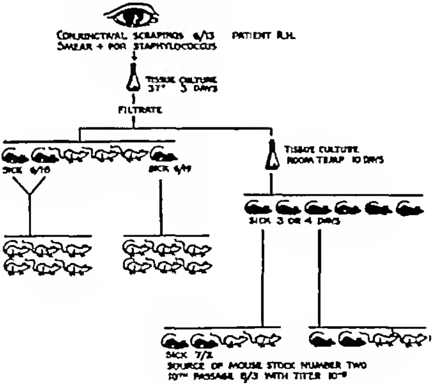

DIAGRAM 2

DIAGRAMS 1 and 2 From the points designated as sources of mouse stock 1 and 2 an agent was obtained which was consistently infectious for mice, after a definite incubation period the mice exhibited a characteristic chain of symptoms which, unlike the transient syndrome seen previously, in all cases led to death. Almost all the injected animals were affected in this manner and there were no convalescents.

Later, the patient stated that his vision had improved about two months after the onset of the disease. No further details were available

Note. The clinical appearance of this patient closely conformed to the criteria of epidemic keratoconjunctivitis considered pathognomonic at the Ophthalmological Institute of Presbyterian Hospital The actual diagnosis was made by Dr Phillips Thygeson

The conjunctival scrapings taken from patient E L on Mar 18 were bacteriologically sterile, and contained large mononuclear cells (Fig 1) According to the procedure followed in the cases studied previously, the scrapings were immediately put into 1 cc. of Simms buffered salt solution (5), and within half an hour, 0 1 cc of this material was injected intraperitoneally and 0 03 cc intracerebrally into mice, each animal being injected by both routes As can be seen in Diagram 1, two of the three mice which received these scrapings showed symptoms within 2 days The symptoms were similar to those seen in the mice which had been injected with conjunctival scrapings from other cases of epidemic keratoconjunctivitis, as described earlier in this report. Brain and spleen from one of these two sick animals were injected intracerebrally (0 02 cc) into six unweaned mice, intracerebrally (0 03 cc.) and intraperitoneally (0 1 cc.) into three adult mice, and intracerebrally into two monkeys and one rabbit In addition to the intracerebral injections the rabbit and one of the monkeys received an emulsion of brain and spleen tissue rubbed into the scarified surface of their right eyes None of these animals showed symptoms

However, the picture was entirely different in the case of the six mice which had received emulsion of brain and spleen tissue from the other of the original two sick animals Two animals in this group became sick in 3 days, two were found dead in 7 days and were discarded The two mice with symptoms were sacrificed, and their brains were pooled and emulsified This emulsion was injected intracerebrally (0 02 cc.) into seven unweaned mice, intraperitoneally (0 1 cc.) and intracerebrally (0 03 cc.) into six adult mice, and passed into a sterile tissue culture of embryonic mouse brain and serum ultrafiltrate As is evident from the diagram, the only animal to show symptoms was one of the adult mice, which became sick in 2 days The infection could be maintained for only one more passage The tissue culture, which had also received the brain emulsion from the mouse which was sick on Mar 23, was incubated for 3 days at 37°C At the end of that time, ground up cells plus the supernatant fluid of the culture were injected intraperitoneally (0 1 cc) and intracerebrally (0 03 cc.) into each of six mice The symptoms these mice subsequently developed were more severe than the symptoms which had appeared in any of the previously injected animals, which, it will be remembered, had received either human conjunctival scrapings or emulsion of brain and spleen from sick mice Two of these mice died The remaining animals, all of which were ill, became the source of *mouse* stock 1, now in its 66th passage.

It should be emphasized that from the point in the scheme of isolation designated as "mouse stock" (Diagram 1), which was reached after passage through tissue culture, the activity of the infectious agent in mice was such as to suggest an increase in potency Whereas on previous occasions, when passage of human eye material from mouse to mouse resulted in a progressively diminished infectivity, now *all* of the

with culture fluid, and filtered through a Berkefeld N filter When injected intra cerebrally (0 03 cc.) and intraperitoneally (0 1 cc.) the filtrate produced symptoms in three of six mice. However, the symptoms could not be transmitted to other mice The filtrate was also passed to a tissue culture which was kept at room temperature for 10 days When this tissue culture was emulsified and injected intracerebrally (0 03 cc.) into six mice, they all became ill within 3 or 4 days Two of these animals were sacrificed, and brain emulsion from each was passed into four mice by intra cerebral inoculation. In each case, two of the four injected mice showed symptoms, and the sick animals became the source of *mouse stock* 2 This mouse stock, now in its 15th passage has a titer of 10^{-5}

The second isolation was not maintained in tissue culture, since the original culture became contaminated in the ice box, and it appeared advisable to concentrate our efforts on the first stock cultures.

Thus, in two instances, it was necessary to pass material from patients through tissue culture and thence into mice, before stable strains of an infec tious agent were isolated which could be studied in laboratory animals If this procedure was not followed, symptoms were not consistently produced in mice. The point of enhanced pathogenicity following the tissue culture passage has been designated as "mouse stock" and from this point death followed in 24 hours the appearance of symptoms No convalescent animals, there fore, were obtained. Throughout this study titrations have been estimated on the basis of the final dilution of virus capable of killing a majority of the mice injected with it The titer is essentially a 50 per cent end point, but it is based on mortality rather than on morbidity That these two agents belonged to the group of virus infections soon became evident, when it was found that they failed to grow in cell-free media, in synthetic media, or in 10 per cent serum broth, when routine examination of the animal tissue proved it to be bacteriologically sterile, when dark field examination of the various media was negative, and when it was found that the agents could be filtered without difficulty It now remained to study the activity of the virus in laboratory animals, and to establish a specific relationship between the isolated agent and epidemic keratoconjunctivitis.

Behavior of the Virus in Laboratory Animals

Once the virus had become adapted by way of tissue culture to mice, its activity in laboratory animals was readily defined The host range investigated included mice rabbits, monkeys, rats, and guinea pigs The most susceptible host, and certainly the most convenient one to study, was the white Swiss mouse. Consequently more information is available concerning the action of the virus in this animal than in others Whereas both strains have been studied in mice, only the virus of the first isolation has been followed in other hosts.

Pathogenicity for Mice—The adapted virus has been consistently patho-

animals injected with the "stock mouse virus" became ill with much more severe symptoms, and, unless sacrificed for subpassage, died True, the incubation period remained the same (2 to 3 days), but the effect in mice was more definitely a disease entity in that a dependable sequence of *illness* and *death* took place

½ cc. of the emulsified tissue culture was also transferred to subcultures, which were incubated at 37°C for 3 days At the end of this incubation period, 0 03 cc of the subculture material was injected intracerebrally into each of four mice, causing illness and death in all of them A further 0 5 cc. transfer was made to two series of tissue cultures One of these was incubated at 37°C for 3 days, the other was kept at room temperature for 7 days The 37°C cultures were discontinued when the combined intracerebral (0 03 cc.) and intraperitoneal (0 1 cc.) injections failed to infect mice The series kept at room temperature, however, has been passed to fresh cultures every 7 days It is consistently infectious for mice, and is the source of *culture* stock 1, now in its 31st generation

Since both the stock culture and passage viruses will be discussed later, it might be well at this point to deal with the second isolation

Case History 2 —Patient R H , aged 58, had been treated at the clinic for about 1 month for an infected chalazion His general health was not very good, and he appeared to be in a state bordering on malnutrition He was first seen by us on June 13, 1942 His clinic report is as follows —

June 5, 1942 Edema of the lids, along with some chemosis of the conjunctiva, was noticeable

June 6 The conjunctiva, markedly injected and with a follicular reaction, had the appearance characteristic of epidemic keratoconjunctivitis

June 8 In the morning some secretion was observed, with mucous shreds in the lower cul-de-sac. Culture of secretions taken on this day showed *Staphylococcus aureus* The preauricular gland was enlarged and tender

June 11 Marked photophobia and lacrimation were present

June 13 Additional complications were added to the above picture anorexia, weakness, pain in the left lower chest, temperature of 101°F The general symptoms might have been quite independent of the eye affection

June 15 Multiple punctate corneal opacities were observed

Aug 6 During the past month and a half little change was seen Conjunctivitis with one corneal subepithelial infiltrate near center was present Preauricular gland was still tender, but questionably palpable

Aug 24 Cornea was unchanged

Sept 28 Some improvement had occurred and the cornea was fairly clear

Note The clinical appearance of this patient closely conformed to the criteria of epidemic keratoconjunctivitis considered pathognomonic at the Ophthalmological Institute of Presbyterian Hospital The actual diagnosis was made by Dr Phillips Thygeson

Conjunctival scrapings taken from R H.'s eye on June 13 were put into tissue culture and incubated for 3 days at 37°C (Diagram 2) Although sterility tests showed the presence of staphylococci in the culture, the tissue was ground, diluted

with culture fluid and filtered through a Berkefeld N filter When injected intracerebrally (0.03 cc.) and intraperitoneally (0.1 cc.) the filtrate produced symptoms in three of six mice However the symptoms could not be transmitted to other mice The filtrate was also passed to a tissue culture which was kept at room temperature for 10 days When this tissue culture was emulsified and injected intracerebrally (0.03 cc.) into six mice, they all became ill within 3 or 4 days Two of these animals were sacrificed and brain emulsion from each was passed into four mice by intracerebral inoculation In each case, two of the four injected mice showed symptoms, and the sick animals became the source of *mouse* stock 2 This mouse stock, now in its 15th passage, has a titer of 10^{-4}

The second isolation was not maintained in tissue culture, since the original culture became contaminated in the ice box, and it appeared advisable to concentrate our efforts on the first stock cultures.

Thus, in two instances, it was necessary to pass material from patients through tissue culture and thence into mice, before stable strains of an infectious agent were isolated which could be studied in laboratory animals. If this procedure was not followed, symptoms were not consistently produced in mice. The point of enhanced pathogenicity following the tissue culture passage has been designated as "mouse stock" and from this point death followed in 24 hours the appearance of symptoms No convalescent animals, therefore, were obtained Throughout this study titrations have been estimated on the basis of the final dilution of virus capable of killing a majority of the mice injected with it The titer is essentially a 50 per cent end-point, but it is based on mortality rather than on morbidity That these two agents belonged to the group of virus infections soon became evident, when it was found that they failed to grow in cell free media, in synthetic media, or in 10 per cent serum broth, when routine examination of the animal tissue proved it to be bacteriologically sterile, when dark field examination of the various media was negative, and when it was found that the agents could be filtered without difficulty It now remained to study the activity of the virus in laboratory animals, and to establish a specific relationship between the isolated agent and epidemic keratoconjunctivitis.

Behavior of the Virus in Laboratory Animals

Once the virus had become adapted by way of tissue culture to mice, its activity in laboratory animals was readily defined. The host range investigated included mice rabbits, monkeys, rats, and guinea pigs The most susceptible host, and certainly the most convenient one to study, was the white Swiss mouse. Consequently more information is available concerning the action of the virus in this animal than in others. Whereas both strains have been studied in mice, only the virus of the first isolation has been followed in other hosts

Pathogenicity for Mice.—The adapted virus has been consistently patho-

genic for mice, and within a definite incubation period produces symptoms that lead to death The dilution activity of the virus has varied from 10^{-5} to 10^{-6} Although no chemical studies have been done, it was noted that the virus could be preserved for at least 3 months in infected mouse brains kept on carbon dioxide ice

Mice intracerebrally injected begin to show symptoms in 2 to 3 days for the lower dilutions, and in 5 to 7 days for the higher dilutions At first the animal is lethargic and has a humped back and a ruffled coat (Fig 2) Death may follow within a few hours after the onset of lethargy, but in a majority of mice, death is preceded by various types of focal nervous symptoms The nervous manifestations include tonic and clonic convulsions, sometimes closely resembling those seen in mice which have received intraperitoneal injections of phenolized material Occasionally, spastic paralyses occur Another symptom which is sufficiently common to be noteworthy, is a peculiar

TABLE I

Distribution of Keratoconjunctivitis Virus in Mice Following Infection by Intracerebral or Intranasal Routes

Route of infection	Tissue tested					
	Brain	Liver	Spleen	Lungs	Kidney	Blood
Intracerebral	10^{-5} 10^{-6}	–	+*	–	–	–
Intranasal	10^{-3}	–	trace	–	–	–

* Organs other than the brain were tested only for the presence of virus

sidling gait seen in about 15 per cent of the infected mice Such animals have a normal gait for 2 or 3 days, and then develop a rotating and sidling gait suggestive of middle ear infection

In adult mice, infection may be transmitted serially by only the intracerebral and, to a lesser extent, the intranasal routes Unweaned mice, however, may be infected by the intraperitoneal route Infection following intranasal injection in adult mice and intraperitoneal injection in unweaned mice, is characterized by an incubation period 1 to 2 days longer than that observed after intracerebral injection In all cases the symptoms are the same

An attempt was made to study the distribution of the virus in infected mice In Table I are the composite data from several such experiments It is clear that in the case of intracerebrally infected mice, virus could be recovered from brain tissue and, to a slight extent, from the spleen In mice infected intranasally, there was little demonstrable virus in the spleen The simplest explanation for this would be that the virus propagates to a greater degree in mice infected intracerebrally That this is the case is indicated by the differ-

ence in brain titers, the titer in the case of mice infected intracerebrally being 10^{-6} and 10^{-6}, and in the case of mice infected intranasally, 10^{-3}

As was reported previously, the pathological picture in mice is not striking, and, in fact, shows a relatively mild degree of structural change, considering the capacity of the virus to kill mice Because little additional information is as yet available concerning the pathology in mice, the findings are essentially those described in the preliminary report (3) The only variations from normal occurred in the central nervous system, in which lesions were scattered diffusely and irregularly through both the gray and white matter Small inflammatory foci, consisting for the most part of perivascular infiltration by lymphocytes, with occasional polymorphonuclear leukocytes (Figs 3 and 5), are the principal lesions A similar infiltration is found to some extent in the perivascular parenchyma (Fig 4), and occasionally neural elements show degenerative changes (Fig 6) Early proliferation of microglia cells is found.

Pathogenicity for Rabbits —As was noted previously, during the period when the virus was being isolated from man, suggestive symptoms were observed in rabbits injected intracerebrally with material from patients' eyes. However, it was not possible to transmit the infection from rabbit to rabbit, or from rabbit to mouse, and the study of this host was temporarily abandoned When the fixed mouse virus was obtained, its pathogenicity for the rabbit was re examined with the following results A 1 50 dilution of 22nd mouse passage brain emulsion injected intracerebrally into four rabbits produced prostration and death in three of the animals within 9 days. In spite of the long incubation period, the infection appeared to be overwhelming, since the animals showed no symptoms for 8 days, and were then prostrated or dead on the 9th day No focal signs, except for an occasional convulsion, were observed A 1 50 dilution of emulsion of the pooled brains of two of these rabbits was injected intracerebrally into four guinea pigs, four rats, and two rabbits Of these animals, only the two rabbits showed symptoms, the incubation period on this occasion being 11 days The symptoms were similar to those seen previously

That the agent responsible for the symptoms was similar to the fixed mouse virus was shown by the fact that the rabbit brains were bacteriologically sterile and that the agent which infected this second group of rabbits was completely neutralized in mice by known convalescent serum Passage from rabbit to rabbit has produced encephalitic symptoms characterized by the usual type of variable convulsions and focal signs During the short period the virus has been observed in rabbits, the incubation period has remained 7 to 12 days and all 17 injected animals have succumbed to the infection. The rabbit series is now in its fifth passage, and it remains to be seen whether a fixed rabbit strain can be obtained. It should also be mentioned that this apparently successful infection of rabbits followed two failures In one case

where the injected material came from an early mouse passage, no infection was observed in a group of five rabbits injected intracerebrally In a second case, the 19th mouse passage virus was injected intracerebrally into four guinea pigs, four rats, and two rabbits Symptoms could be observed in the rabbits within 7 days, but the *infection could not be transmitted* to other rabbits Again, the guinea pigs and rats remained free from symptoms

Pathogenicity for Monkeys—Portions of the same conjunctival scrapings that had been studied in the first and second isolations of virus from patients E L and R H were injected intraconjunctivally into four monkeys with no effect On several occasions the mouse-fixed virus was injected into the conjunctiva and intracerebrally One monkey developed a brain abscess, three showed no effect whatsoever, and two showed vague nervous symptoms 3 days before death, which occurred in 5 days Brains from these two animals were sterile, and when injected into mice produced symptoms suggestive of the epidemic keratoconjunctivitis virus To these may be added two baby *Macacus rhesus* monkeys, which received mouse virus intraconjunctivally In spite of repeated traumatizing inoculations, the eyes remained entirely free from infection On one occasion, two adult *Macacus rhesus* monkeys injected intraconjunctivally with mouse virus developed a transient but definite catarrhal conjunctivitis In the absence of a preauricular node enlargement and because the condition lasted only 2 days, no definite conclusions could be drawn

Further investigation of monkey susceptibility has been postponed, because of the difficulty in obtaining the animals for experimental purposes

Pathogenicity for Guinea Pigs and Albino Rats—Since its isolation the mouse virus has been injected into 36 guinea pigs by intracerebral, subcutaneous, and intraperitoneal routes None of these animals has at any time shown symptoms, nor have there been any deaths Similar results were obtained when 12 albino rats were injected with the mouse virus It is clear that neither guinea pigs nor albino rats can be infected

Activity of the Virus in Tissue Culture

The serum ultrafiltrate technique has been successfully used for the culture of other viruses (4) This medium, containing a cellular substrate of embryonic mouse brain, was used throughout the present investigation

When the first isolation of the virus had been effected in tissue culture, some difficulty was encountered in maintaining serial subcultures On three occasions a subculture inoculum consisting of ground-up cells plus culture fluid was compared with one of culture fluid alone Freshly prepared tissue cultures which received only the culture fluid proved to be non-infectious regardless of the incubation conditions, whereas the flasks receiving ground up tissue continued to be infectious for mice Consequently, the routine inoculum

from culture to culture has consisted of both ground up tissue and culture fluid Also, because of the instability of the 37°C series, greater dependence has been placed upon room temperature cultures, and this series is now in its 31st generation, having been subcultured every 6 or 7 days.

TABLE II

Tissue Culture Potencies of Mouse Keratoconjunctivitis Virus Measured by Intracerebral Injection of Mice

Culture generation	Mouse titer
3rd to 10th (no titration done)	Virus present
11th	10^{-2}
12th*	10^{-2}
	10^{-3}
13th	10^{-2}
14th	10^{-4}
15th	10^{-2+}
16th	10^{-3}

* Two tests done.

TABLE III

*Growth of Mouse Keratoconjunctivitis Virus in Tissue Culture at Room Temperature**

Period of incubation	Mouse titer
days	
2	0
3	0
4	10^{-1}
5	$10^{-2.5}$
6	10^{-2}
7	$10^{-2.5}$
8	$10^{-2.5}$

* The 13th subculture was the source of virus for this experiment.

Perhaps the simplest explanation for the necessity of passing both tissue and fluid menstruum from infected to fresh cultures is the relatively low potency of the cultures. As can be seen from Table II, the culture titer in mice has rarely exceeded 10^{-2} While the tissue cultures seem effective as a link in adapting the virus from human to mouse, and in building up a level of potency which would consistently cause symptoms in these animals, once established in an animal host the virus attains a greater potency than in the artificial medium It can be seen from Table III, that in subculture no virus is demonstrable until the 4th day of incubation at room temperature, and a

terol appeared promptly with overeating, the increase being roughly proportional to the rate of weight gain But as overeating and gaining weight continued, the serum cholesterol concentration did not tend to change further

Since determinations of serum S_f 12–20 lipoproteins were made only at the middle and end of the overnutrition period the change in S_f 12–20 lipoproteins in the last ten weeks was tested for correlation with increase in caloric intake and with gain in body weight No significant correlation was found in either case During this latter half of the overnutrition period serum cholesterol increase between periods was likewise unrelated to the degree of overeating or weight gain

The correlation analysis described attempted to answer the question—what change do overeating and the maintenance of a positive calorie balance produce in serum cholesterol and S_f 12–20 lipoprotein concentrations? It is also pertinent to ask—is there a tendency for men who are overweight to have unusually high concentrations of serum cholesterol or S_f 12–20 lipoproteins?

Among middle-aged men examined by the Laboratory of Physiological Hygiene, 162 maintained constant relative body weight within 5 per cent for 4 years In this group of men who varied widely in relative obesity but all of whom were close to calorie equilibrium, there was no correlation between serum cholesterol concentration and relative body weight (correlation coefficient = 0 02, total number of tests = 648) The overweight men had no higher serum cholesterol concentration than the underweight men In the men who were overeating and in positive calorie balance, the relationship was different Using 79 tests on 20 men during the period of overfeeding, the coefficient of correlation was 0 35 between serum cholesterol concentration and relative body weight This value is small but it is statistically significant ($P = 0\ 004$) Among these men the most overweight individuals tended to have the highest serum cholesterol concentrations

Serum S_f 12–20 lipoprotein was similar to serum cholesterol in relation to relative weight In the men who were overeating, 31 tests on 19 men gave a coefficient of correlation of 0 40 between S_f 12–20 lipoprotein and relative body weight Although the coefficient is higher, the probability judgment is less decisive in this case ($P = 0\ 025$) because the number of tests was smaller Like serum cholesterol, the S_f 12–20 fraction showed no correlation with relative weight in the men who were stable in body weight, 73 tests on 72 men resulted in a correlation coefficient of 0 08

After the overfeeding experiment was finished the men were allowed to eat as they pleased without any guidance Eighteen months later 19 of the 20 men were again observed Seven men had lost more than 10 kg since the end of overfeeding and 6 of these were among the 9 men who had gained over 10 kg in the experiment They showed an average fall of 20 mg of cholesterol and 40 mg of S_f 12–20 lipoprotein per 100 ml of serum (ranges − 57 to − 8 and − 82 to − 10, respectively) from their values at the end of the overfeeding period Eleven of the former subjects showed smaller weight changes since the overfeeding period, and this group included 7 of the 9 men who had gained least in the experiment Their serum cholesterol and S_f 12–20 concentrations showed a statistically insignificant tendency to rise slightly (averages of + 15 and + 10 mg per cent, respectively) from their values at the end of the experiment

DISCUSSION

What caused the observed significant increases in serum cholesterol? It has been repeatedly observed that, in calorie equilibrium, a major change in the proportion of calories provided as mixed food fats tends to produce corresponding changes in the concentration of cholesterol and of S_f 12–20 lipoprotein in the serum (9, 11–13)

In the present experiment the proportion of calories provided by fats was slightly *decreased* during the overfeeding (37 per cent versus 39 per cent), so the present observations might seem discordant with these other findings However, it will be observed that the absolute amount of fat consumed was increased by an average of 22 gm per day and such a change in fat consumption under conditions of calorie equilibrium may be expected to produce a rise in serum cholesterol concentration (14, 15)

Consideration of these facts might suggest that the serum cholesterol level tends to reflect the total fat ingestion, probably because cholesterol is a necessary part of the lipoprotein fat-transport system in the blood In calorie equilibrium this load of fat metabolism is directly related to the

proportion of the total calories consumed as fats but in positive calorie balance the absolute fat intake may be a better measure of the fat metabolism load. But this explanation, without further elaboration fails to account for the fact that men who are physically active tend in comparison with less active men in the same population, to have an elevated absolute fat intake but not elevated blood cholesterol values (16) Moreover, when young men greatly increase their energy expenditure by exercise and maintain calorie equilibrium by increasing the diet the serum cholesterol does not rise in spite of a considerable increase in fat consumption (17)

However allowance must be made for the fact that when the total rate of energy metabolism is increased, there is normally a parallel increase in the circulatory rate so that the *rate* of fat transport will be increased even though there is no rise in *concentration* of the fat or lipoprotein in the blood Accordingly it would seem that all of the present data are in harmony with the concept that, other things being equal, the serum cholesterol concentration is determined by the fat transport load per unit of circulation imposed on the blood At calorie equilibrium this is determined by the proportion of the calories presented as fats and this relationship is not altered by increasing the energy level of intake if calorie equilibrium is maintained by increased exercise which normally also involves a proportionate increase in circulatory rate. In positive calorie balance, the fat transport load is obviously increased even if the character of the diet is constant Indeed, if fat storage is taking place, that portion of the fat synthesized from carbohydrate in the liver also adds to the transport load so we might expect some serum cholesterol rise even on a reduced fat diet during the active phase of gaining weight. But if the calorie excess and the weight gain steadily continue, there is no further increase in the fat transport load and the serum cholesterol should remain constant at its newly raised level Finally if calorie equilibrium is now achieved and obesity is steady, the serum cholesterol should be expected to approximate that characteristically associated with the proportion of fat in the diet at calorie equilibrium regardless of whether this equilibrium means steady obesity, steady 'normality' or steady leanness

The fat transport of relevance would seem to be that from intestine to liver and from liver to fat depots These avenues are active in overnutrition and in high fat ingestion and are inactive in undernutrition and on a low fat or fat free regiment. The transport of fat from the depots to the liver and of fat metabolites from the liver to the muscles for burning is less obviously related to serum cholesterol In undernutrition the serum cholesterol level usually falls though much fat is being transported away from the adipose depots

The increase of S_f 12–20 lipoprotein which occurred in the last half of the overfeeding period involved the transfer of only about one-fortieth of the cholesterol of the plasma from one lipoprotein class to another There is no apparent reason for the difference but this lipoprotein class seems to have exhibited a slower or longer continued response to the increase in food intake than the preponderant cholesterol bearing fractions

SUMMARY

Twenty physically healthy schizophrenic men increased their calorie intakes without changing their physical activity The diet was substantially constant and fully adequate in proteins and vitamins at all times the extra calories being provided by adding carbohydrates and a small amount (about one-third of the extra calories) of mixed fats to the standard diet. Thus the total fat intake increased though the proportion of calories from fats fell slightly Average calorie increases for 20 weeks ranged from 8 per cent to 39 per cent and these produced weight gains from 2 5 to 22.2 kg the average gain being about 0 5 kg per week.

The average total serum cholesterol concentration rose 20 mg per 100 ml during the first 5 weeks of overeating and then remained substantially constant at the same elevated level during the next 15 weeks though weight gain continued at the same rate as during the first five weeks The rise in serum cholesterol concentration in the various individuals tended to be proportional to the rate of weight gain.

The concentration of the S_f 12–20 lipoprotein fraction in the serum measured in the ultracentrifuge tended to increase from the tenth to the twentieth week of overeating though the total serum cholesterol remained constant

Weight gain tended to be associated with increase in the circulating plasma and blood volume during the first weeks of overeating with no further change thereafter

A hypothesis as to the role of cholesterol in fat transport is presented which seems to explain 1) The increase in serum cholesterol on a high fat diet and in overnutrition with a positive calorie balance, 2) The stability of the serum cholesterol during calorie equilibrium or in a steady state of continuing overnutrition, 3) The failure of serum cholesterol to rise when diet calories and energy expenditure are both increased in parallel, and 4) The decrease in serum cholesterol on a low fat diet and *in negative calorie balance*

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URINARY HEMOGLOBIN EXCRETION AND RENAL CIRCULATORY DYNAMICS A STUDY OF THE EFFECT OF L-NOREPINEPHRINE IN THE DOG[1, 2]

By WILLOUGHBY LATHEM[3] AND ALVIN D BENJAMIN[4]

(*From the Department of Internal Medicine Yale University School of Medicine and the Veterans Administration Hospital West Haven Conn*)

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The passage of protein molecules across capillary walls is governed by the characteristics of the molecule and the membrane and appears to occur by a process of both filtration and diffusion. From a study of these processes within the isolated perfused hind limb of the cat (2 3) and from mathematical considerations and other studies (2 4) it has been suggested that the transcapillary transport of protein and the degree of molecular sieving of protein molecules across membranes during ultrafiltration may be influenced by concentration gradients of protein between filtrate and filtrand and by the rate of filtration of water through the capillary wall. Moreover, it has been suggested that these processes may be influenced by factors which modify the level of hydrostatic pressure within the capillary (2, 5 6)

Within the kidney these processes have not been subjected to extensive study That intraglomerular hydrostatic pressure may modify the rate of filtration of protein molecules has been suggested by experiments in which renin, which presumably elevates intraglomerular pressure, has been shown to accelerate the urinary excretion of plasma proteins (7 8) and hemoglobin (9) A change in pressure has also been invoked to account at least in part for the augmentation of protein clearances following the administration of serum albumin intravenously to patients with renal disease (5) Of molecular sieving within the kidney and of the influence of changes in glomerular filtration rate, little is known

In the present investigation these factors were examined in normal, anesthetized dogs The rate of urinary excretion and renal clearance of hemoglobin were studied under conditions in which renal hemodynamics and glomerular filtration rate were modified by infusions of the adrenal medullary hormone l norepinephrine.

METHOD

A study of the effects of l norepinephrine was made in 20 female mongrel dogs weighing 7 to 20 kg. All animals were deprived of food, but not water for 24 hours prior to the test. Anesthesia was induced with sodium pentobarbital, 30 mgm. per kg intravenously and was maintained by the administration of 100 mgm. of this drug at intervals throughout the study An indwelling needle was placed in a femoral artery Priming and sustaining infusions of creatinine and p-aminohippurate were administered intravenously for measurement of glomerular filtration rate and renal plasma flow respectively Along with these substances solutions of dog hemoglobin were administered intravenously in amounts sufficient to maintain a relatively constant arterial plasma level of hemoglobin of from 212 to 504 mgm. per cent, values well above the renal threshold (10) Priming doses consisted of from one to two grams of hemoglobin in 50 ml. of 5 per cent dextrose and distilled water The sustaining infusion contained hemoglobin in a concentration of from 430 to 760 mgm. per cent and was made up in 5 per cent dextrose and distilled water This was administered at a constant rate of from 1 to 2 mgm. of hemoglobin per minute.

After allowing 30 minutes for equilibration of these substances, the urine, collected by urethral catheter was discarded and the bladder was washed out with distilled water and emptied by manual compression after the introduction of air to facilitate emptying Three consecutive 10 to 20-minute control clearance periods were then obtained. Following this an infusion of l norepinephrine (16 μg per ml.) was administered intravenously at a rate sufficient to elevate mean arterial blood pressure, measured with a mercury manometer 20 to 50 mm. Hg This

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[2] The results of this study have been published in abstract form (1)

[3] Present address Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

[4] This work was submitted to the Faculty of Medicine, Yale University in partial fulfillment of requirements for the degree of Doctor of Medicine.

TABLE I

*Urinary hemoglobin excretion and renal hemodynamics during the infusion of l norepinephrine**

Dog No	Wt. Kg	Procedure	BP mm Hg	HR /min	GFR ml/min	RPF ml/min	FF	P_{Hb} mg %	$U_{Hb}V$ mg/min	C_{Hb} ml/min	$\frac{C_{Hb}}{C_{cr}}$ %
3	10	Control	114	120	54	112	.482	250	3.04	1.22	2.28
		Infusion	165	72	41	76	.541	305	4.22	1.38	3.42
7	17	Control	94	140	35	70	.501	249	2.30	0.92	2.65
		Infusion	140	180	21	34	.620	348	4.22	1.12	5.71
8	20	Control	104	120	98	207	.471	212	2.11	0.93	0.99
		Infusion	140	120	85	147	.587	223	4.18	1.87	2.18
9	7	Control	85	160	48	87	.552	504	4.58	0.91	2.09
		Infusion	115	140	20	43	.473	501	3.71	0.74	3.71
10	7	Control	110	160	31	135	.230	394	0.72	0.18	0.61
		Infusion	135	128	39	118	.350	470	2.28	0.48	1.26
11	8	Control	85	130	27	131	.203	354	1.90	0.54	2.04
		Infusion	130	100	8	23	.427	403	1.71	0.43	5.69
12	12	Control	95	140	31	92	.339	317	2.18	0.68	2.20
		Infusion	130	128	26	82	.316	379	4.40	1.16	4.46
13	13	Control	120	132	32	117	.276	308	2.35	0.77	2.44
		Infusion	150	—	17	50	.355	391	6.97	1.79	10.36
14	9	Control	100	132	24	76	.320	336	2.66	0.89	3.83
		Infusion	130	168	13	36	.428	397	3.47	0.91	8.22
15	7	Control	90	124	23	53	.435	395	3.12	0.88	3.90
		Infusion	110	160	15	33	.466	410	4.30	1.06	7.68
16	15	Control	90	156	44	111	.406	244	2.71	1.12	2.56
		Infusion	120	160	38	91	.415	283	3.28	1.16	3.70
19	13	Control	105	132	23	56	.434	244	3.62	1.50	6.54
		Infusion	135	120	12	30	.397	310	3.16	1.03	8.83
31	16	Control	100	112	98	209	.473	460	2.07	0.45	0.47
		Infusion	150	100	88	177	.496	523	3.52	0.68	0.77
32	12	Control	100	92	70	108	.648	301	1.69	0.56	0.80
		Infusion	130	140	62	99	.630	405	3.02	0.75	1.21
17	9	Control	98	148	38	106	.358	285	1.94	0.69	1.81
		Infusion	140	120	27	59	.470	321	2.40	0.76	2.91
		Recovery	70	134	32	75	.422	331	2.79	0.85	2.70
18	9	Control	95	148	37	100	.375	273	2.26	0.83	2.22
		Infusion	130	120	18	43	.499	300	2.76	0.92	7.59
		Recovery	90	140	25	63	.416	301	3.19	1.06	4.08
25	9	Control	95	100	62	94	.655	246	3.96	1.65	2.73
		Infusion	140	132	56	90	.615	323	3.62	1.12	2.04
		Recovery	90	98	57	98	.576	331	5.79	1.74	3.02
33	13	Control	110	120	44	117	.396	477	2.67	0.56	1.28
		Infusion	160	100	32	57	.555	598	5.50	0.92	2.91
		Recovery	75	138	41	90	.459	593	4.25	0.71	1.71
34	10	Control	110	84	69	104	.663	345	1.10	0.32	0.47
		Infusion	160	136	59	90	.655	419	2.40	0.57	0.98
		Recovery	75	138	66	142	.471	406	2.01	0.50	0.76
35	12	Control	120	144	32	82	.394	341	1.25	0.38	1.16
		Infusion	160	128	19	35	.553	450	2.83	0.63	3.55
		Recovery	—	—	19	59	.334	441	1.36	0.30	1.74

* All values are averages of three determinations L-norepinephrine was administered intravenously during infusion periods Abbreviations are as follows BP = mean arterial blood pressure HR = heart rate, beats per minute, GFR = glomerular filtration rate (creatinine clearance) RPF = renal plasma flow (PAH clearance), FF = filtration fraction (GFR/RPF) P_{Hb} = plasma hemoglobin concentration $U_{Hb}V$ = urinary hemoglobin excretion, C_{Hb} = clearance of hemoglobin C_{Hb}/C_{cr} = hemoglobin-creatinine clearance ratio

required between 4 and 50 µg of l norepinephrine per minute. In most animals the amount necessary to maintain arterial pressure at the desired level increased progressively throughout the test. After elevation of the arterial pressure for approximately 15 minutes the urine was discarded and three consecutive 10 to 20-minute clearance periods were obtained during the pressor response to l norepinephrine. In each of six animals (17 18, 25 33 34 and 35) three recovery periods were obtained immediately following cessation of the infusion of l norepinephrine.

Urinary and plasma concentrations of hemoglobin were determined by the method of Evelyn and Malloy (11) Creatinine concentrations in urine and plasma were assayed by the method of Bonsnes and Taussky (12) and PAH levels were determined by the method of Smith, Finkelstein, Aliminosa, Crawford, and Graber (13) Hemoglobin saline solutions were prepared according to the method of Amberson, Jacobs, Hisey and Monke (14) Solutions of hemoglobin prepared in this manner were dialyzed against normal saline at 8 C for 48 hours. After filtration through a Seitz filter the solution was stored in a sterile container at 8 C for periods not exceeding two weeks.

RESULTS

The results of the effects of l norepinephrine on renal hemodynamics and hemoglobin excretion are presented in Table I and in Figures 1 to 6 An illustrative experiment is presented in Figure 1 (dog 33)

Systemic circulatory response

During the infusion of l norepinephrine the arterial blood pressure increased from 20 to 50 mm Hg with a mean change of 38 mm Hg Considerable variation was encountered in the pressor response to l norepinephrine. Some animals required as much as 50 µg per minute to maintain the pressure within this range whereas others re-

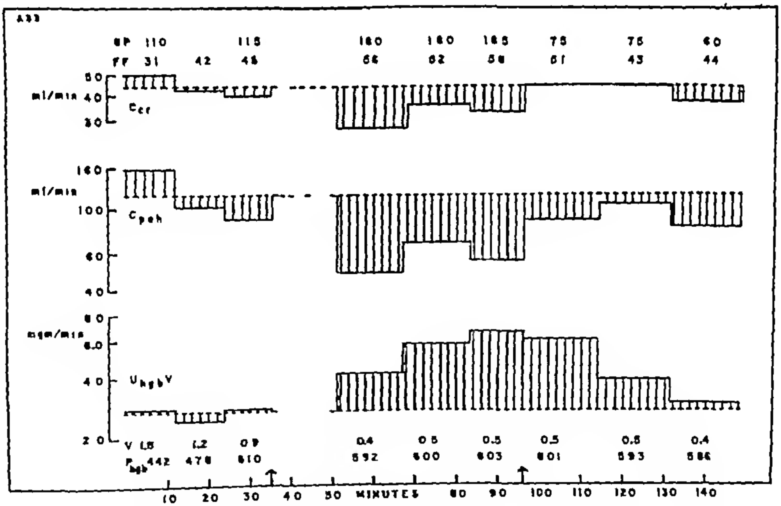

FIG. 1 THE EFFECT OF L-NOREPINEPHRINE ON RENAL FUNCTION AND URINARY HEMOGLOBIN EXCRETION (DOG 33)

Glomerular filtration rate (C_{cr}-creatinine clearance) renal plasma flow (C_{PAH}), urinary hemoglobin excretion ($U_{Hb}V$) and mean arterial blood pressure (BP) were determined before, during and following the administration of l norepinephrine. The drug was given intravenously during the time noted between the arrows. The urine obtained during the first 15 minutes after beginning infusion was discarded because of intrarenal delay Urinary hemoglobin excretion increased in association with a rise in arterial pressure and plasma hemoglobin concentration (P_{Hb}, at bottom) and a fall in glomerular filtration rate and renal plasma flow The filtration fraction increased. Urine flow (V) diminished. After the infusion was discontinued all values returned towards control levels.

TABLE I

*Urinary hemoglobin excretion and renal hemodynamics during the infusion of l-norepinephrine**

Dog No.	Wt. Kg	Procedure	BP mm Hg	HR min	GFR ml./min	RPF ml./min	FF	P_{Hb} mgm %	$U_{Hb}V$ mgm/min	C_{Hb} ml/min	$\frac{C_{Hb}}{C_{cr}}$
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		Infusion	140	180	21	34	.620	348	4.22	1.12	5.71
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		Infusion	140	120	85	147	.587	223	4.18	1.87	2.18
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10	7	Control	110	160	31	135	.230	394	0.72	0.18	0.61
		Infusion	135	128	39	118	.350	470	2.28	0.48	1.26
11	8	Control	85	130	27	131	.203	354	1.90	0.54	2.04
		Infusion	130	100	8	23	.427	403	1.71	0.43	5.69
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13	13	Control	120	132	32	117	.276	308	2.35	0.77	2.44
		Infusion	150	—	17	50	.355	391	6.97	1.79	10.36
14	9	Control	100	132	24	76	.320	336	2.66	0.89	3.83
		Infusion	130	168	13	36	.428	397	3.47	0.91	8.22
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		Infusion	130	120	18	43	.499	300	2.76	0.92	7.59
		Recovery	90	140	25	63	.416	301	3.19	1.06	4.08
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		Recovery	75	138	66	142	.471	406	2.01	0.50	0.76
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		Infusion	160	128	19	35	.553	450	2.83	0.63	3.55
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Urinary and plasma concentrations of hemoglobin were determined by the method of Evelyn and Malloy (11) *Creatinine concentrations in urine and plasma* were assayed by the method of Bonsnes and Taussky (12) and PAH levels were determined by the method of Smith, Finkelstein, Aliminosa, Crawford, and Graber (13) Hemoglobin-saline solutions were prepared according to the method of Amberson, Jacobs Hisey, and Monke (14) Solutions of hemoglobin prepared in this manner were dialyzed against normal saline at 8°C for 48 hours. After filtration through a Seitz filter the solution was stored in a sterile container at 8°C for periods not exceeding two weeks.

RESULTS

The results of the effects of l norepinephrine on renal hemodynamics and hemoglobin excretion are presented in Table I and in Figures 1 to 6 An illustrative experiment is presented in Figure 1 (dog 33)

Systemic circulatory response

During the infusion of l norepinephrine the arterial blood pressure increased from 20 to 50 mm Hg with a mean change of 38 mm. Hg Considerable variation was encountered in the pressor response to l-norepinephrine Some animals required as much as 50 μg per minute to maintain the pressure within this range whereas others re-

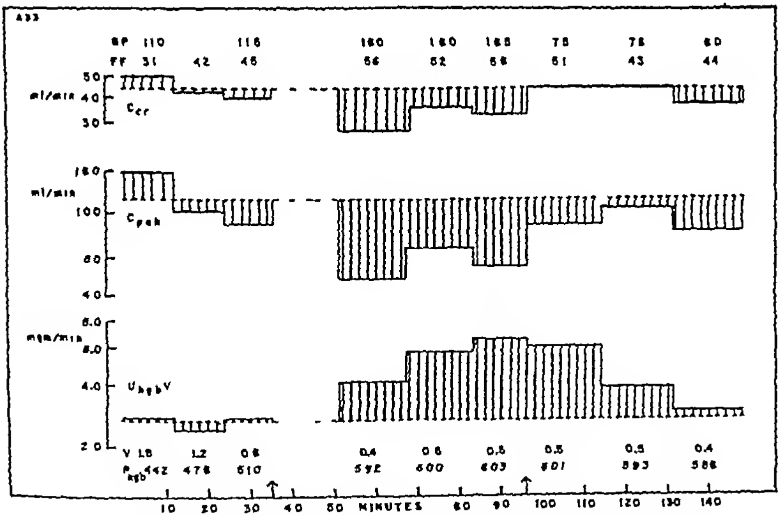

FIG. 1. THE EFFECT OF L-NOREPINEPHRINE ON RENAL FUNCTION AND URINARY HEMOGLOBIN EXCRETION (DOG 33)

Glomerular filtration rate (C_{cr}-creatinine clearance) renal plasma flow (C_{PAH}) urinary hemoglobin excretion ($U_{Hb}V$) and mean arterial blood pressure (BP) were determined before, during and following the administration of l norepinephrine. The drug was given intravenously during the time noted between the arrows. The urine obtained during the first 15 minutes after beginning infusion was discarded because of intrarenal delay Urinary hemoglobin excretion increased in association with a rise in arterial pressure and plasma hemoglobin concentration (P_{Hb}, at bottom) and a fall in glomerular filtration rate and renal plasma flow The filtration fraction increased. Urine flow (V) diminished. After the infusion was discontinued all values returned towards control levels.

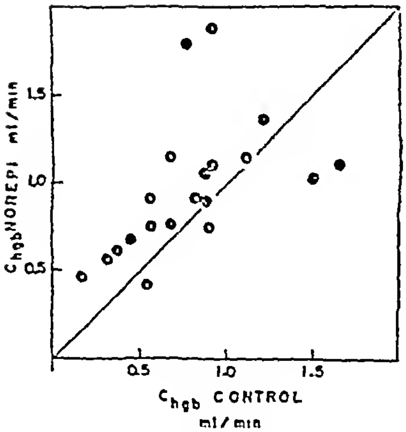

FIG 2 THE EFFECT OF L-NOREPINEPHRINE ON THE RENAL CLEARANCE OF HEMOGLOBIN

The averaged values for the clearance of hemoglobin during the control periods are plotted against the clearances of this protein during the pressor response to l norepinephrine. The clearance of hemoglobin increased in 14 studies, decreased in 4, and remained unchanged in 2

quired as little as 4 μg per minute After the infusion was discontinued the blood pressure returned promptly to control levels or below Two dogs (17 and 33) became hypotensive at this time (Table I) In most animals the cardiac rate decreased 20 to 30 beats per minute during infusion of l-norepinephrine, in others, a tachycardia with

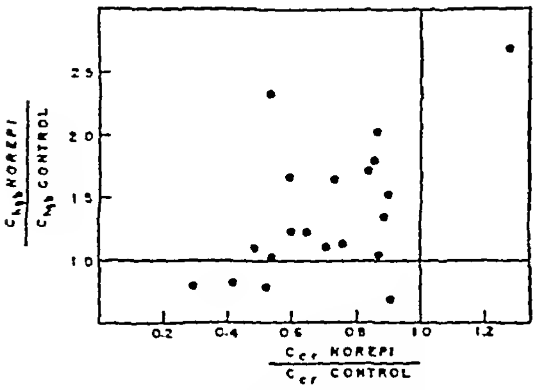

FIG 3 THE RELATIONSHIP BETWEEN THE CHANGES IN THE CLEARANCE OF HEMOGLOBIN AND THE CLEARANCE OF CREATININE DURING THE PRESSOR RESPONSE TO L-NOREPINEPHRINE

The ratio of the infusion to the control values of the clearance of hemoglobin is plotted against the changes in the creatinine clearance. The clearance of hemoglobin tended to increase during the infusion of l norepinephrine except in those instances when the reduction in the creatinine clearance exceeded 50 per cent of control values

varying degrees of irregularity of cardiac rhythm was observed

Renal hemodynamics

During control observations prior to the infusion of l-norepinephrine the mean renal plasma flow for the entire group of 20 animals was 100 ml per 10 kg body weight per minute, a value less than that reported (15) for normal dogs under basal conditions—135 ml per 10 kg body weight per minute The mean renal filtration fraction (GFR/RPF) was 0 43, a value higher than that reported (15) for normal animals — 0 32 These differences may be attributed to the administration

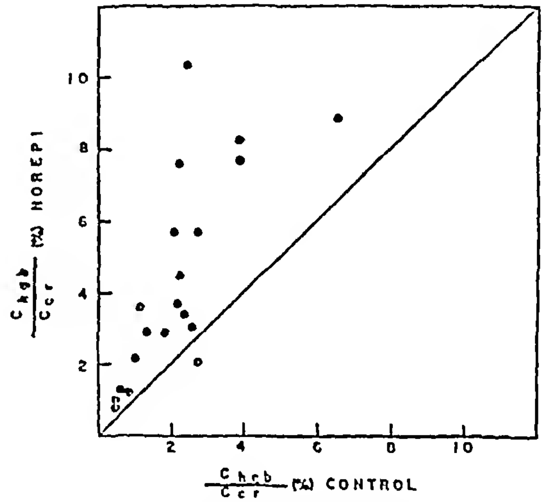

FIG 4 THE EFFECT OF L-NOREPINEPHRINE ON THE HEMOGLOBIN-CREATININE CLEARANCE RATIO

The control values of the hemoglobin creatinine ratios (C_{Hb}/C_{Cr}) are plotted against these ratios obtained during the infusion of l-norepinephrine. The hemoglobin clearance increased relative to the creatinine clearance in all studies but one.

of hemoglobin, which elicits intrarenal vasoconstriction (16) The mean glomerular filtration rate was 41 ml per 10 kg body weight per minute, a figure not significantly different from normal values (15)

During infusion of l-norepinephrine renal plasma flow (RPF, Table I) decreased in all dogs but one (dog 25) The mean renal plasma flow at this time was 63 per cent of control values, with a range of 16 to 96 per cent of control levels Glomerular filtration rate (GFR, Table I) diminished more than 10 per cent in 17 dogs (range of

change, 11 to 70 per cent) remained unchanged in two (dogs 25 and 31) and increased in one (dog 10) For the entire group the mean value of GFR during the infusion of l norepinephrine was 71 per cent of control levels There was no correlation between the magnitude of blood pressure elevation and the degree of change in GFR. The correlation between the rate of infusion of l norepinephrine and the change in GFR was significant ($r = -446$ $p < 05 > 01$) The filtration fraction (FF Table I) increased in 11 dogs, decreased in 4 and remained unchanged in 5 On cessation of infusion of l norepinephrine RPF, GFR, and FF returned towards control values (recovery period, Table I)

Urinary hemoglobin excretion ($U_{Hb}V$, Table I Figure 1) increased during the pressor response to l norepinephrine in 17 studies, decreased in 2 (dogs 9 and 11) and remained unchanged in one (dog 25) The mean excretory rate at this time was 166 per cent of control values with a range of 81 to 316 per cent. These changes in excretion were usually but not invariably accompanied by an increase in the concentration of hemoglobin in plasma. In 17 animals the plasma level of hemoglobin increased 10 to 40 per cent (mean change, 20 per cent), in three (dogs 8 9 and 25) the plasma level did not change. The rise in plasma concentration may be attributed to the hemoconcentrating effects of l norepinephrine (17 18) and, in some instances, to a greater rate of administration than of excretion of hemoglobin.

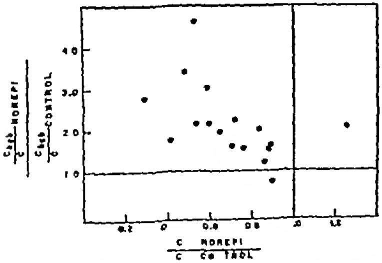

FIG. 5 THE RELATIONSHIP BETWEEN THE CLEARANCE OF CREATININE AND THE HEMOGLOBIN-CREATININE CLEARANCE RATIO DURING THE PRESSOR RESPONSE TO L NOREPINEPHRINE

The ratio of the infusion to the control values of the hemoglobin-creatinine clearance ratios (C_{Hb}/C_{Cr}) is plotted against the ratio of the infusion to control values of the clearance of creatinine (C_{Cr}) As C_{Cr} diminished, the clearance of hemoglobin increased relative to creatinine. In one study C_{Hb}/C_{Cr} increased as C_{Cr} rose.

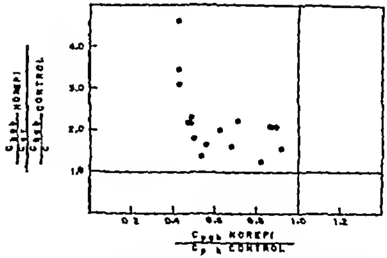

FIG. 6. THE RELATIONSHIP BETWEEN THE CLEARANCE OF PAH AND THE HEMOGLOBIN-CREATININE CLEARANCE RATIO DURING THE PRESSOR RESPONSE TO L-NOREPINEPHRINE

The ratio of the infusion to the control values of the hemoglobin-creatinine clearance ratios (C_{Hb}/C_{Cr}) is plotted against the ratio of the infusion to the control values of the PAH clearance. As the clearance of PAH diminished, the clearance of hemoglobin increased relative to creatinine.

The renal clearance of hemoglobin (C_{Hb}, Table I) showed similar directional changes (Figure 2) However, since the concentration of hemoglobin in plasma increased in most studies, the percentage change in hemoglobin clearance was less than that of hemoglobin excretion. C_{Hb} increased in 14 animals decreased in 4 (dogs 9, 11, 19 and 25) and remained unchanged in 2 (dogs 14 and 16) This variability may be attributed in part to the magnitude of the reduction in the volume of glomerular filtrate, since the clearance of hemoglobin tended to fall when GFR was reduced more than 50 per cent (Figure 3) The mean C_{Hb} during the infusion of l norepinephrine was 137 per cent of control values with a range of 69 to 267 per cent.

On cessation of the infusion the excretion and clearance of hemoglobin returned towards control values in three studies (dogs 33, 34 and 35) and increased in three (dogs 17, 18 and 25) The increase in dog 25 was spurious since this animal developed gross hematuria during the recovery periods

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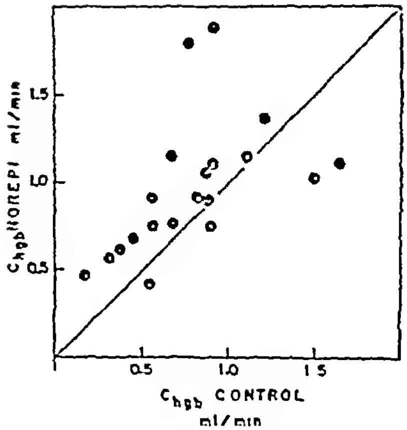

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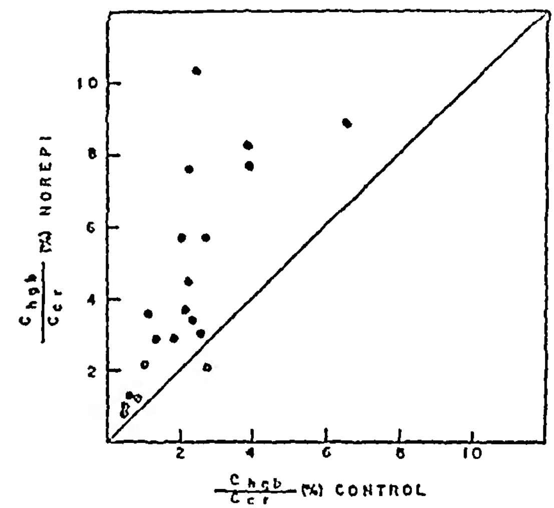

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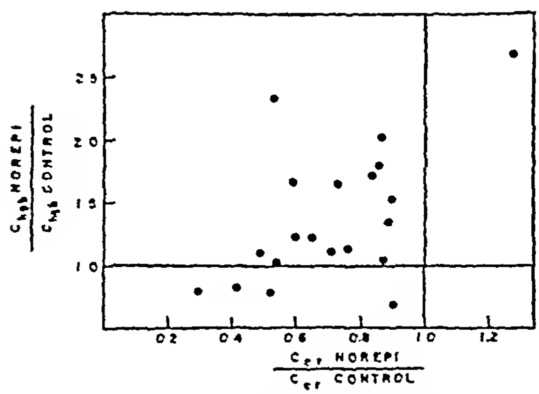

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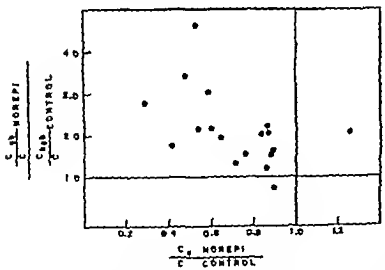

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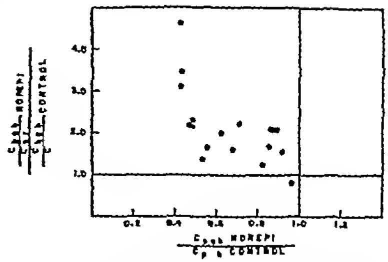

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The hemoglobin-creatinine clearance ratio (C_{Hgb}/C_{Cr}, Table I) increased in every study but one (dog 25) (Figure 4) The mean value during the

administration of l-norepinephrine was 211 per cent of control levels with a range of 75 to 463 per cent The correlation between the changes in this ratio and GFR was of borderline significance ($r = -0.436$, $p < .10 > .05$) (Figure 5)[5] The correlation between the changes in C_{Hgb}/C_{Cr} and RPF was significant ($r = -0.590$, $p < .01$) (Figure 6) On cessation of the infusion of l-norepinephrine the hemoglobin-creatinine clearance ratio returned towards control levels in all animals except dog 25, which developed gross hematuria

DISCUSSION

Hemoglobin molecules circulating in plasma are excreted in the urine at a rate which is determined by *the rate of transport of these molecules across* the glomerular membrane and by the capacity of the renal tubular cells to abstract this protein from glomerular filtrate during the process of urine formation The present study suggests that either one or both of these processes concerned in hemoglobin excretion may be modified during the pressor response to l-norepinephrine The increase in urinary excretion and in the renal clearance of hemoglobin elicited by the infusion of l-norepinephrine may be attributed to either an increase in transglomerular transport of hemoglobin or to a decrease in tubular reabsorption, or to both Although the precise mechanism of this excretory response was not established, the magnitude of the changes suggests that alterations in tubular reabsorption alone were not responsible In dogs 12, 13, and 33 the increment in hemoglobin excretion exceeded 2 0 mgm per minute (by 2 2, 4 62, and 2 83 mgm per minute, respectively), the approximate maximal rate of tubular reabsorption of hemoglobin in dogs the size of those employed in the present study (10) Hence, the augmented hemoglobin excretion may be attributed, at least in part, to a more rapid rate of transfer of hemoglobin molecules into glomerular filtrate Whether changes in tubular reabsorption also occurred is not known

The mechanism of this alteration in transglomerular transport is not clear Since the concentration of hemoglobin in glomerular filtrate rises in a linear manner as plasma hemoglobin concentration increases (10, 19), the increment in plasma concentration usually elicited in the present study by the infusion of l-norepinephrine undoubtedly contributed to this increase in transport in most studies However, the increase in plasma level was inconstant (dogs 8 and 9) and was insufficient in most instances to account for the magnitude of the increment in the clearance of hemoglobin and in the hemoglobin-creatinine clearance ratio[6] Hence other factors appear to have been operative as well to augment the glomerular transport of this protein

That alterations in glomerular filtration rate may have initiated these changes in transport must

[5] When the one study (dog 10) in which the creatinine clearance increased is omitted from these calculations, the correlation between GFR and C_{Hgb}/C_{Cr} is significant ($r = -0.539$, $p < .02 > .01$)

[6] *This view can be illustrated by theoretical calculations* of the change in the clearance of hemoglobin and in the hemoglobin-creatinine clearance ratio to be expected by a 20 per cent increase in the plasma hemoglobin concentration, the average increment observed in the present study during the infusion of l norepinephrine

(a) Assuming the following values plasma hemoglobin concentration, 3 0 mgm per ml, creatinine clearance, 40 ml per minute, glomerular permeability to hemoglobin, 5 per cent of the creatinine clearance (18), and Tm_{Hgb}, 2 0 mgm per minute

$$\text{Filtered Hgb} = 3.0 \times 40 \times .05 = 6.00 \text{ mgm/min}$$

$$\text{Reabsorbed Hgb} = 2.00 \text{ mgm/min}$$

$$\text{Excreted Hgb} = 4.00 \text{ mgm/min}$$

$$C_{Hgb} = \frac{4.00}{3.00} = 1.33 \text{ ml/min}$$

$$C_{Hgb}/C_{Cr} = \frac{1.33}{40} = .033$$

(b) Assuming a 20 per cent increase in P_{Hgb} and no change in glomerular permeability or tubular reabsorption

$$\text{Filtered } H_{gb} = (3.0 + 0.6) \times 40 \times .05 = 7.20 \text{ mgm/min}$$

$$\text{Reabsorbed } H_{gb} = 2.00 \text{ mgm/min}$$

$$\text{Excreted } H_{gb} = 5.20 \text{ mgm/min}$$

$$C_{Hgb} = \frac{5.20}{3.60} = 1.44 \text{ ml/min}$$

$$C_{Hgb}/C_{Cr} = \frac{1.44}{40} = 0.36$$

Per cent change in C_{Hgb} = 8 per cent.

Per cent in C_{Hgb}/C_{Cr} = 9 per cent.

These changes are less than those determined experimentally (average experimental change in C_{Hgb}, 37 per cent and in C_{Hgb}/C_{Cr}, 111 per cent) Hence an increase in plasma hemoglobin concentration of 20 per cent cannot alone account for the observed alterations

be considered in view of the relationship between protein transport and filtration rate as formulated in the theory of molecular sieving According to this theory, the concentration of protein in capillary filtrates increases as the rate of formation of filtrate diminishes owing to differences in the diffusion characteristics of protein and water (creatinine) molecules (2-4) Although GFR characteristically fell in the present study the role of this reduction is obscure. That the excretory response to l norepinephrine may have been *modified* by changes in GFR is evident in Figure 3 which shows that the typical increase in hemoglobin clearance was abolished by marked decrements of GFR[7] But whether adjustments of GFR *initiated* the excretory response to l norepinephrine in accordance with the concept of molecular sieving is not clear In one study (dog 10) the clearance of hemoglobin increased (both in absolute terms and relative to creatinine) in association with an increase rather than a decrease in GFR. Moreover, the relationship between filtration rate and protein transport is uncertain owing to doubt concerning the mechanism of the reduction of GFR in the dog during the vasoconstrictive response to l norepinephrine. If as has been suggested (20), filtration diminishes as a result of selective cessation of glomerular activity in a portion of the nephron population, the theory of molecular sieving as outlined cannot account for the changes in hemoglobin clearance elicited in the present study Since under these circumstances, filtration would be unaltered in those nephrons contributing to urine formation the relationship between the transglomerular transport of hemoglobin and creatinine would not change in these nephrons and the hemoglobin-creatinine clearance ratio would remain constant. If however, GFR decreases by a generalized partial reduction of filtration in active nephrons, or if variable alterations of filtration rate in residual, active glomeruli occur during nephron exclusion, the transport of hemoglobin across the glomerular membrane of functioning nephrons might increase relative to creatinine in accordance with the theory of molecular sieving and the clearance of hemoglobin would rise relative to creatinine. Until however, the precise mechanism of the reduction of GFR is established the role of these changes in filtration rate cannot be evaluated.

Studies of the effects of plasma volume expansion on proteinuria (5) and of the effect of renin on the excretion of hemoglobin and plasma proteins in rabbits and rats (7-9) have suggested that the transport of protein molecules across the glomerular membrane may be conditioned by the level of intracapillary hydrostatic pressure Renin, which has hemodynamic effects in the kidney similar to those elicited by l norepinephrine, is thought to elevate intraglomerular pressure by vasomotor adjustments of the efferent and afferent arterioles (8) This rise in pressure, it has been suggested (8) accelerates the filtration and/or diffusion of protein across the glomerular membrane by mechanisms which are not clear but which may be related to stretching of the glomerular membrane (5) Whether l norepinephrine acts in a similar manner is not known A rise in intraglomerular pressure would be expected to elevate glomerular filtration rate relative to renal plasma flow (an increase in the renal filtration fraction), a functional pattern which is elicited by both renin and l norepinephrine But whether this relationship between filtration rate and plasma flow actually reflects adjustments of intraglomerular pressure is not clear (15) Moreover if nephron exclusion occurs during the administration of l norepinephrine, a variable and uncertain relation between flow and filtration may exist in active and inactive nephrons and hemodynamic interpretation of the filtration fraction would be impossible Hence the changes in protein transport elicited in the present study cannot be evaluated in terms of adjustments of intraglomerular pressure. Whether the level of hydrostatic pressure genuinely influences protein transport has not been unequivocally established The precise role of this factor in the kidney and other sites remains to be determined

That hemodynamic adjustments within the kidney may have altered the transport of hemoglobin across the glomerular membrane independently of alterations in glomerular filtration rate and intraglomerular pressure is possible. The relationship

[7] The decrease in the clearance of hemoglobin under these circumstances may be attributed to the marked reduction in the volume of glomerular filtrate. Since the clearance of hemoglobin increased relative to creatinine in these instances the factors tending to augment the transglomerular transfer of hemoglobin appear to have been operative in these as in the other studies.

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THE RENAL LESION IN EPIDEMIC HEMORRHAGIC FEVER [1]

By JEAN OLIVER and MURIEL MACDOWELL

(*From the Renal Research Unit Overlook Hospital Summit N J*)

(Submitted for publication June 21 1956 accepted September 5 1956)

Structural and functional derangements in the kidney during the course of Epidemic Hemorrhagic Fever run as a continuing thread of cause and effect through a complex of physiological and biochemical disturbances that has perhaps no parallel among the acute infectious diseases So closely knit are these renal aberrations in the fabric of the clinical syndrome that investigators have found it impractical to disentangle the element of renal failure from the related phenomena of shock hypotension or hypertension and electrolyte or water imbalance and so simply state as their conclusion that renal complications are present in all examples of the disease and at every stage of its progress (1)

The general nature of the renal lesion has been established In its clinical aspects it is an example of acute renal failure with the typical characteristics of proteinuria oliguria and late diuresis From the physiological viewpoint, the investigations of Froeb and McDowell (2) and of Syner and Markels (3) have demonstrated similar disturbances of renal blood flow which occur either as a part or at times independently of the general circulatory collapse which so frequently ensues in the early *phases of the disease.*

The pathological alterations that occur in the kidneys have been described by the Russian investigators who first recognized the disease in eastern Siberia (4) [2] and in later studies by the Japanese (4) More recently Hullinghorst and Steer (6) Steer (7) and Lukes (8) have reported on material obtained during epidemics among American troops in Korea Though the resemblance of the histological picture to that which is found in other forms of the acute renal necrosis associated with traumatic and toxic injury has impressed pathologists certain characteristics of the structural lesions of EHF are so distinctive that in the absence of a demonstrable etiological agent they have become the final criterion for its diagnosis

A general discussion and detailed analysis of the *clinical* physiological and biochemical derangements that occur in EHF have appeared in a Symposium published under the editorial direction of Dr David P Earle (9) Against this background an attempt will now be made to integrate the development of the structural aspects of the renal lesion with the progress of its functional disturbances

MATERIAL AND METHODS

The clinical data and pathological material of this study have been taken from the records of the Commission of Epidemic Diseases and the collections of the Armed Forces Institute of Pathology

Thirty nine fatal cases of EHF which occurred during the Spring and Fall epidemics of 1952 and 1953 among American troops in Korea have been examined An analysis of thirty four of these appears in the Symposium (9) the case numbers of this earlier study have been retained and though much of its substance has been incorporated into our report further pertinent information may be derived from this source, in particular from Table VIII page 636.

The distribution of the cases in regard to the phase of the disease in which the individual died was as follows

Hypotensive phase		19
(Primary shock	12)	
(Transition shock	7)	
Oliguric phase		10
Diuretic phase 1 to 16 days of diuresis		9
Convalescence, 149 days from onset		1
		39

[1] This work was done under the auspices of the Commission on Hemorrhagic Fever Armed Forces Epidemiological Board, and was supported by the Office of the Surgeon General of the Army and in part by a research grant (H 1515 C2) from the National Heart Institute, of the National Institutes of Health Public Health Service and the Life Insurance Medical Research Fund.

[2] Though the earlier reported cases of EHF were limited to Manchuria and eastern Siberia, later observations have shown that a very similar disease occurs in the Balkans and Czechoslovakia. The mild "epidemic nephropathy" observed in Finland, Sweden and Norway resembles EHF in some ways but an identity has not been proved (5)

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MATERIAL AND METHODS

The clinical data and pathological material of this study have been taken from the records of the Commission of Epidemic Diseases and the collections of the Armed Forces Institute of Pathology

Thirty nine fatal cases of EHF which occurred during the Spring and Fall epidemics of 1952 and 1953 among American troops in Korea have been examined. An analysis of thirty four of these appears in the Symposium (9) The case numbers of this earlier study have been retained, and though much of its substance has been incorporated into our report further pertinent information may be derived from this source in particular from Table VIII page 636

The distribution of the cases in regard to the phase of the disease in which the individual died was as follows

Hypotensive phase		19
(Primary shock	12)	
(Transition shock	7)	
Oliguric phase		10
Diuretic phase 1 to 16 days of diuresis		9
Convalescence 149 days from onset		1
		39

[1] This work was done under the auspices of the Commission on Hemorrhagic Fever Armed Forces Epidemiological Board, and was supported by the Office of the Surgeon General of the Army and in part by a research grant (H 1515 C2) from the National Heart Institute of the National Institutes of Health, Public Health Service, and the Life Insurance Medical Research Fund.

Though the earlier reported cases of EHF were limited to Manchuria and eastern Siberia, later observations have shown that a very similar disease occurs in the Balkans and Czechoslovakia. The mild epidemic nephropathy" observed in Finland, Sweden and Norway resembles EHF in some ways but an identity has not been proved (5)

The method of study was as follows. The autopsy protocols and clinical records were first reviewed. Sections of all the tissues from each autopsy were then examined for whatever relation they might bear to the renal lesion and the sections of kidneys studied in detail by the conventional methods of histological examination. In the material of this preliminary survey 10 per cent formalin and Zenker's solution had been the fixative and hematoxylin and eosin the stain; in subsequent examinations the Masson stain, iron hematoxylin and eosin, Acid Fast Green and certain histochemical procedures were used.

After this examination had established the general nature of the renal lesion, microdissection of the kidney tissue as previously described (10) was done to observe the detail and topographical relations of abnormalities in the nephron. The dissected nephrons were stained with iron hematoxylin and camera lucida drawings or photomicrographs were made for permanent record. As will appear in the descriptions of the structural alterations, a continuous comparison was made during the progress of the work between the appearance of the lesions as they were revealed by the two methods of examination.

Selected examples illustrating the course and development of the pathological alterations appear in our illustrations. It is perhaps unnecessary to point out that considerations of expense and space have made necessary a considerable restriction in the presentation of our findings. During the dissection of each case hundreds of nephrons were examined, many were drawn by means of the camera lucida and a lesser number photographed at magnifications of up to 200 times. Of the latter only typical examples can be published and at great reduction; the original of Plate I, for example, is composed of 97 individual 4 by 5 microphotographs which, when assembled to show the complete nephron, cover a space of 4 by 9 feet; much detail is therefore lost in the process of publication.

In the exposition of our findings the nomenclature used by clinical investigators to designate the course of the disease has been adopted. A brief review of the syndrome of EHF may orient the reader for the more detailed description of the physiological, clinical and pathological correlations that are to follow.

THE CLINICAL SYNDROME OF EHF

During all but the first of the five successive phases, Febrile, Hypotensive, Oliguric, Diuretic and Convalescent, into which the clinical course of EHF has been divided, disturbances of renal activity are prominent. The Febrile Phase is characterized by the usual fever, chills, headache and nausea of an acute infectious disease, and ends around the fifth day by defervescence.

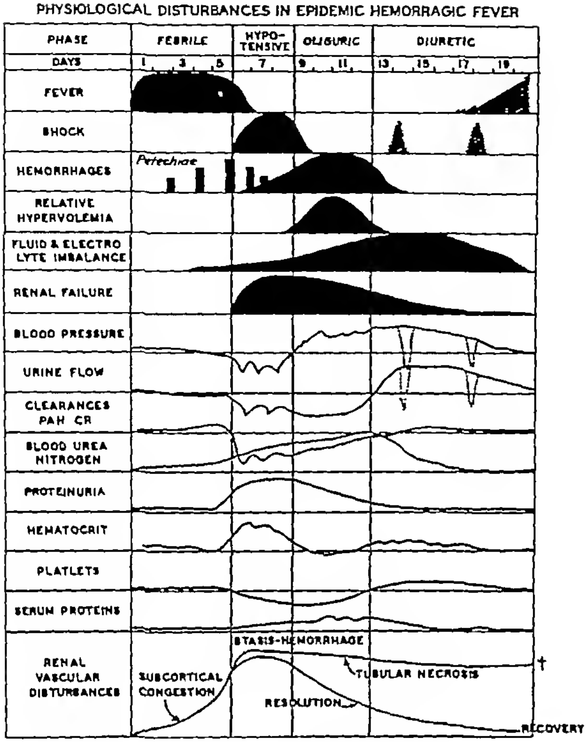
With passage into the Hypotensive Phase the earlier manifestations of disturbance in the peripheral circulation, such as the intense flush of the face and the injection of the pharynx, become definitive in the appearance of petechiae. Concomitantly there occurs a rise in the hematocrit which along with direct determinations of decreased circulating volume suggests that plasma fluid is leaking through damaged capillaries into tissue spaces. As a result the blood pressure falls and shock ensues.

These conditions persist during the Hypotensive Phase in which death from primary shock accounted for one-third of all fatalities. Proteinuria is now massive and the urinary output is irregularly decreased. Blood urea nitrogen rises and, with passage from the Hypotensive Phase, the oliguria previously irregularly manifested, becomes permanently established to mark the third stage of the disease.

The elevated hematocrit of the Hypotensive Phase has now decreased toward normal, at times abruptly but more usually requiring one to three days before it becomes stabilized at its original level. During this Transition Period from the Hypotensive to the Oliguric Phase, a circulatory collapse of a nature somewhat different from the "primary shock" of the earlier period may occur in "transition shock"; the observation that total serum proteins do not change as the hematocrit falls and direct measurements of an increasing circulatory volume suggest that fluid previously lost to the extravascular spaces is returning.

During the Phase of Established Oliguria which follows, hypertension is commonly observed, but a more remarkable circulatory disturbance during this period is the development in certain cases of a *relative* hypervolemia along with hemorrhagic phenomena during which the complications of pulmonary edema, hemorrhage or convulsions may be fatal.

After 3 to 5 days of oliguria the Diuretic Phase begins; a daily output as high as 18 L has been recorded. During this phase abnormalities in electrolyte and water balance may develop and a condition of "limited homeostasis" be established in which secondary shock and pulmonary edema occur. Judging from the evidence of increasing clearances there then follows an improvement in the renal circulation which gradually reaches normal in the following weeks. The BUN has fallen, proteinuria has disappeared and in the Convalescent Period most patients after two months



TEXT FIG. 1 COURSE OF CLINICAL AND LABORATORY OBSERVATIONS IN SEVERE EPIDEMIC HEMORRHAGIC FEVER

Modified from Sheedy and his associates (1)

can concentrate their urine to a specific gravity of 1 023 or better

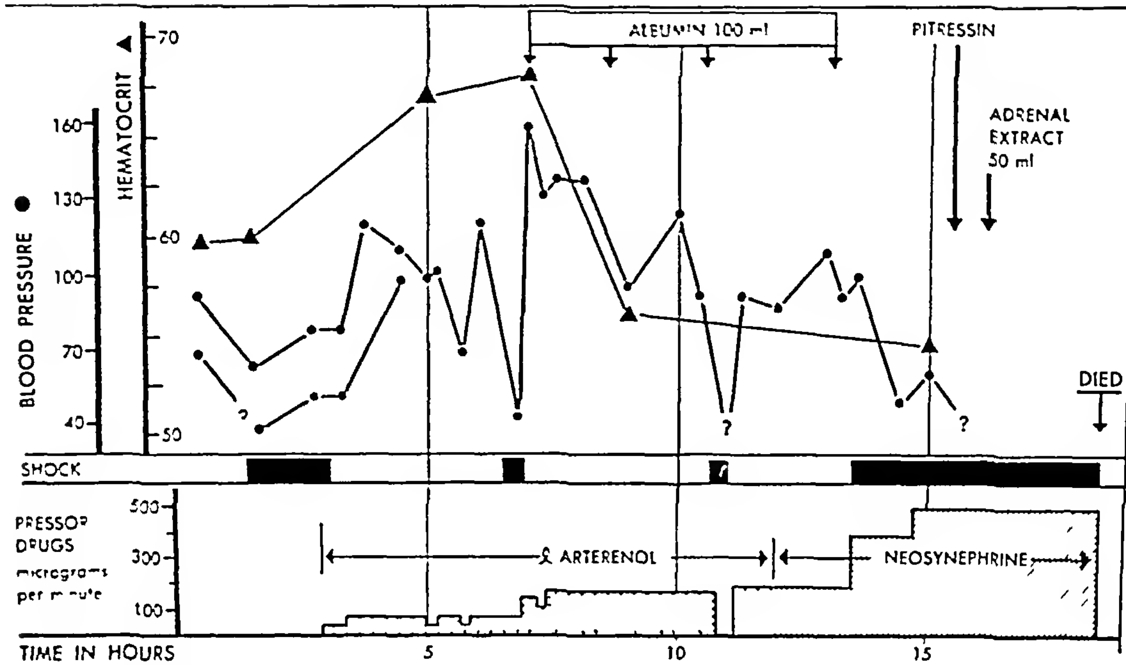
Text Figure 1 which is a modification of Figures 2 and 3 of Sheedy and his co-workers (9) shows the clinical manifestations and laboratory measurements of EHF in graphic form The lowest level illustrates the occurrence and relative intensity of certain pathological phenomena that were observed in the kidney and will be explained by our later descriptions

Although as previously stated the renal lesion is to be described as it develops in the consecutive clinical stages of the disease the procedure of presenting the individual cases has been to arrange them in a series based on the evolution of the pathological lesions As these lesions are of varied nature involving vascular interstitial and parenchymal reactions which may develop at differing rates of intensity such a series can be only approximate in arrangement.

THE RENAL LESION IN THE FEBRILE AND HYPOTENSIVE PHASE

(The Prehemorrhagic Stage of Subcortical Congestion)

The earliest view of the renal lesion in EHF encountered in this series was obtained in Case



TEXT FIG. 2. CASE 38—DEATH IN PRIMARY SHOCK PHASE OF HEMORRHAGIC FEVER

Note initial response to continuous intravenous pressor therapy but increasing requirements during subsequent course and death after 18 hours observation despite 400 cc concentrated human albumin, Pitressin® and adrenal cortical extract. Note also shock at eleventh hour when l-arterenol infusion infiltrated into subcutaneous tissue. Blood pressure could be measured only by palpatory method after fifth hour. Marked retroperitoneal edema was present at autopsy.

Text Figures 2, 4, 6, 7 and 9 and their legends are from the Symposium on Epidemic Hemorrhagic Fever (9) and are published by permission of author and publisher from the American Journal of Medicine, 1954, 16, 617.

38 who died on the fourth day of his illness. After three days of severe headache, chills, fever, nausea and vomiting he entered the hospital with a temperature of 103°, a blood pressure of 100/70 mm Hg, a 1+ proteinuria and a hematocrit of 59 per cent. Physical examination showed intense injection of the conjunctivae and soft palate but no petechiae were present. During the examination he suddenly vomited and collapsed; his blood pressure fell to 58/0 mm Hg. In spite of continuous treatment* including the infusion of 4 units of serum albumin which produced intermittent re-establishment of blood pressure (Text Figure 2) he expired in shock 18 hours after admission. During this period, with a fluid intake of 2864 cc he passed 415 cc of urine; the last sample before death showed 2+ protein and the terminal BUN was 42.3 mg per cent.

At autopsy no petechiae were present. There was a considerable amount of retroperitoneal edema lightly stained with the dye T-1824 that had been injected for determination of blood volume. The kidneys were not swollen (175 to 200 gm.) and the prosector noted that though the cortex was *more sharply differentiated from* the medulla than normally, there was not the intense congestive zone which he was accustomed to find as typical of EHF. There were, however, the usual hemorrhages in the right atrium and an extensive hemorrhagic infiltration of the anterior lobe of the pituitary.

On histological examination the glomerular

* In this and the cases which follow only that therapeutic procedure has been given for which there is evidence that it may produce a structural alteration in the kidney, namely the administration of concentrated human serum albumin (1 unit = 100 cc 25 per cent salt poor serum albumin). Many individuals also received whole blood transfusions, glucose and nor-adrenalin infusions and occasionally Dextran. A summary of these procedures may be found elsewhere (9).

tufts were essentially normal the capillaries contained red blood cells in normal numbers and there was precipitated granular material in Bowman s space Cross sections of proximal convolutions in the cortex were lined with their typical epithelium which showed some evidence of apical swelling of its cells (Figures 1 and 2) the cytoplasm of these cells was normal The lumens of cross sections of ascending limbs in the medullary rays and the distal convolutions in the cortex were dilated and appeared empty (Figure 2)

In the subcortical medullary zone were scattered areas of dilatation of intertubular vessels which were crowded with discrete red blood cells (Figure 3) The thin outlines of the intact walls of these vessels were clearly evident and there was no escape of blood into the interstitial tissue (Figure 4) In these areas of congestion the epithelium and basement membranes of the straight tubules terminal proximal convolutions and ascending limbs of Henle s loop though somewhat compressed were well preserved Throughout the deeper portions of the medulla were irregularly distributed patches of intertubular edema but little evidence of congestion and none of hemorrhage (Figure 5) The tubules of the collecting system were normal and contained few casts

Microdissections of the renal tissue showed essentially normal configurations in the nephrons except for some slight pressure effect on those terminal segments of the proximal convolutions and ascending limbs of Henle s loop which passed through areas of congestion

Plate I A to F shows a complete nephron stained with iron hematoxylin Apart from the localized effects just noted the general configurations of this nephron are so slightly disturbed that it may serve as a control example of the appearance of a normal nephron prepared by our technique for comparison with the damaged specimens that are to follow In all the preparations the intense black staining of the proximal convolution is the reaction of the mitochondria which fill the cytoplasm of its epithelial cells in decreasing amount as one departs from the glomerulus these cellular organelles no longer exist in their original rod like form but apparently as a result of the maceration in HCl are resolved into fine granules As in histological preparations stained with iron hematoxylin the nuclei do not stain heavily but appear as light round spots on the dark background of the cytoplasm except in the premitotic phases of regenerative proliferation when their excessive and hyperchromatic chromatin makes them clearly visible It should be recalled that the tubule is being viewed through its entire thickness 60 to 70 μ in the case of the proximal convolution so that cellular detail is of necessity somewhat clouded on the other hand this increase in the absolute amount of tissue under observation at times permits the recognition of cytoplasmic lesions which are not appreciable in thin histological sections Since a familiarity with the microscopic appearance of dissected nephrons is essential to the recognition and the interpretation of the alterations that are to be seen in the abnormal specimens a detailed description of Plate I will be given

The glomerulus due to the bulk of its tissues shows little cellular detail its size and shape are normal however and the relation of the more opaque tuft to Bowman s capsule is evident.

The proximal convolution is of normal configuration and diameter except in its terminal medullary portion which, passing into an area of congestion in the subcortical outer stripe of the outer zone of the medulla is somewhat compressed The cytoplasmic pattern is in general well preserved the normal mitochondrial gradient is shown by the gradual decrease in the intensity of the reaction to iron hematoxylin and the clear vacuolar round nuclei more visible as the intensity of the mitochondrial staining lessens are normally distributed in the tubule wall At *a* in Plate IB the medullary segment of the convolution entered an area of intense intertubular congestion which lay just below the cortex The tubule is definitely narrowed and although its cells are in general well preserved slight irregularities are noted toward the end of the segment and a scattering of intracellular detritus indicates some epithelial damage.

The greater part of the thin portion of Henle s loop though successfully dissected and mounted, was swept away during the process of staining (Plate ID) a short remnant, somewhat dilated remains (Plate IE) and passes abruptly into the thick portion of the limb which continuing to descend forms the loop and turns towards the cortex In the area of congestion lying adjacent

to the compressed terminal segment of the proximal convolution the cellular pattern of the ascending limb is slightly disturbed and there is some deeply stained intracellular detritus. Above this point and extending high into the cortex the ascending limb is moderately dilated; its cellular components appear essentially normal.

The distal convolution (Plate II) is much more and very unevenly dilated so that there is a consequent patchy thinning of its wall which accounts for the irregularity of its staining; there are no coagula or casts in its distended lumen. The juncture of its terminal portion, the connecting tubule, with that of a neighboring nephron is not dilated.

Dissected collecting tubules from cortex to deep medulla were entirely normal; those which passed through the areas of congestion were unaffected by the dilatation of the intertubular vessels which surrounded them.

The nephron shown in Plate I was typical of those which were involved in an area of subcortical congestion and none of these showed more severe alterations. Those which passed outside or between the scattered areas of congestion, at a rough estimate perhaps one-half of all nephrons, showed no visible abnormalities in their structure.

Text Figure 3 is a graphic representation of a renal lobule from this kidney. The nephrons shown are camera lucida tracings of dissected specimens. They and the areas of intertubular congestion are arranged as they were observed to lie in the kidney during the course of dissection and as similar structures and relations appeared in histological sections. For the sake of clarity only a few nephrons are shown and these are widely spaced, but otherwise the figure presents not an interpretive diagram but a reconstruction of actuality.

It is clear that the architecture of the kidney and the topographical relation of its constituent nephrons have not been greatly affected by the vascular disturbance in the subcortical medulla. The dilatation of the distal portions of the nephron lying in the cortex, including ascending limbs of Henle's loop and distal convolutions, is, however definite, in no way striking (Figure 2 and Plate II); this dilatation was associated with a zone of moderate pressure effect from the congestion in the subcortical medulla, a relation that will assume greater significance with the progress of the renal lesion.

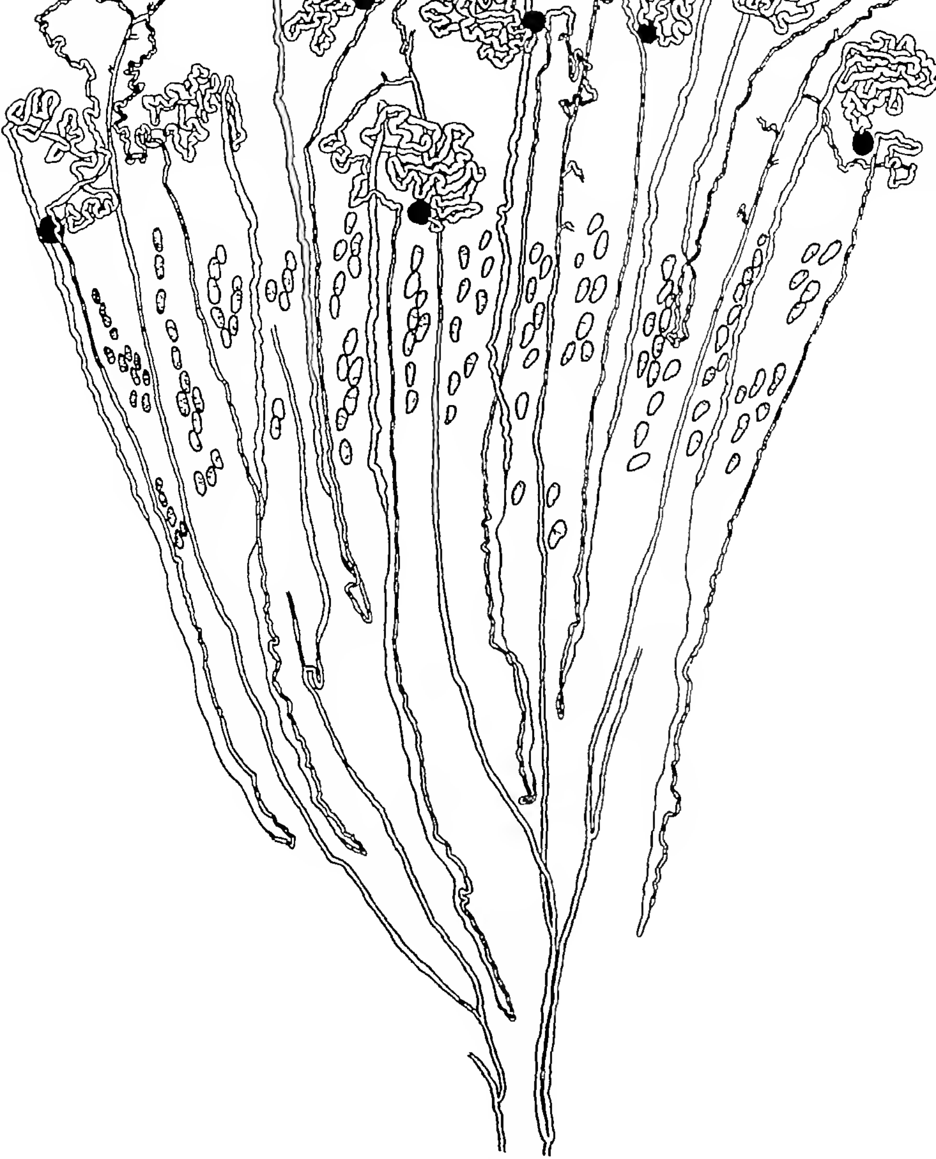
* * * * *

Since the renal lesions become increasingly complex with the progress of the disease, a summary of the structural findings and a discussion of their significance along with a reference to pertinent literature will be given at the end of each clinical period. This procedure may make for reiteration, but will perhaps be less burdensome to the reader than the continuing back reference to minutiae long since described which would be required if critical considerations were postponed to a final conclusion.

The kidney of this individual in whom defervescence was interrupted by sudden death from primary shock may be taken as illustrative of the renal status during the late Febrile Phase at its passage into the Hypotensive Phase. The lesion can be briefly summarized. There is a marked congestive hyperemia localized to the subcortical zone of the medulla, occurring not diffusely but in irregular patchy areas, with no intertubular hemorrhage. There are some slight localized pressure effects of this congestion, both of compression and dilatation, on the tubules of nephrons that by chance pass through it, but no structural evidence of general cellular damage in any of the tubules. The tubular passages are clear; there are few casts.

The correlation of these structural changes with the clinical and physiological data of the period seems reasonably clear; all lines of evidence indicate the beginning of widespread disturbances in the peripheral vascular bed which are characterized first by dilatation and then by increased permeability of capillaries and venules.

In the kidneys the functional effects of these disturbances are reflected in the clearance examinations of Froeb and McDowell (2) and of Syner and Markels (3), who found not a lessening of renal blood flow, as is observed in classical acute renal failure, but either its full maintenance or actual increase. The histological sections show the distribution of this abundant blood flow in the congestive hyperemia of the subcortical medulla. To a consideration of the reason for this localization we shall return in a later description of the progress of the vascular lesion. Neither histo-



TEXT FIG 3 A RENAL LOBULE FROM THE EARLY HYPOTENSIVE PHASE OF EPIDEMIC HEMORRHAGIC FEVER—CASE 38

Except for some dilatation of the distal convolutions the topography of the nephrons is normal There is an irregular intense congestion of the subcortical medulla but no intertubular hemorrhage. Magnification 18 ×

logical sections nor dissections showed frank structural alterations in the nephrons, a finding which correlates with the clinical observation that there was only a moderate degree of proteinuria and elevation of BUN.

It may be concluded therefore from both the structural and functional findings that the vascular bed of the kidney shares in the general disturbance of the peripheral circulation, the clinical evidences of which were vividly present in the intense flush of the skin of the face and chest and mucous membranes. All these phenomena together constitute the first evidences of generalized capillary damage and in the case described they terminated abruptly in primary shock when plasma escaped into the extravascular spaces, a phenomenon indicated by the clinical observation of a rising hematocrit and confirmed by the pathological finding of massive retroperitoneal edema. It is noteworthy that the ultimate lesion of vascular damage was as yet not developed in either the skin or the kidney, for neither petechiae were present nor intertubular hemorrhage. This gravest aspect of the vascular lesion was, however, present in two areas, the right auricle and the anterior hypophysis; the small vessels of these regions are apparently peculiarly susceptible since in every case in this series they were the seat of extensive hemorrhage.

*   *   *   *   *

The remaining cases of those dying in primary shock may be considered to have definitely entered the Hypotensive Phase, a conclusion supported not only by the evolution of the clinical symptomatology but by the progress of the renal lesion.

The first of these, Case 9, entered hospital with a 3+ proteinuria 3½ days after a typical febrile onset. The next morning the hematocrit rose to 61.9 per cent, the blood pressure fell and the patient went into shock; in spite of the administration of 1200 cc. of blood and 3 units of serum albumin he died in shock the morning of the following day. There was no oral intake of fluid and there was a urinary output of 760 cc.; the terminal temperature was 106°, the BUN on the day before death was 16.8 mg. per cent. At autopsy, marked retroperitoneal edema, hemorrhages in the right auricle and an extensive hemorrhagic infiltration in the anterior pituitary were present. The kidneys were swollen; their medullae are described as "reddish purple."

Histological examination showed an intense, patchy engorgement of the subcortical zone of the medulla. In certain areas the capillaries between the terminal proximal convolutions and ascending limbs of Henle's loop were dilated to the diameter of the adjacent tubules and crowded with red blood cells which in some vessels were discrete and in others packed and fused into a hyaline mass (Figure 6). In spite of this distention the walls of the vessels were not greatly thinned and their intact endothelial cells were plainly visible. The tubules surrounded by these vessels were compressed by the resulting tension and their epithelium showed a moderate degree of protoplasmic disturbance.

Not only were the small vessels of the outer medulla distended with red cells, but the capillaries of the swollen glomerular tufts throughout the cortex were greatly dilated and packed with red blood cells (Figure 7). There was considerable precipitated granular material in Bowman's space. Between the cross sections of the cortical proximal convolutions the intertubular capillaries were not particularly prominent. Both the large veins and the arteries of the corticomedullary junction were, however, engorged with blood.

Whereas the epithelium of the proximal convolutions of the previously described case was essentially normal, there was now present diffusely throughout the cortex a definite swelling of the epithelial cells, and their protoplasm showed irregular variations in density without, however, the occurrence of definite vacuoles. Although the epithelial protoplasm was increased in its granularity, no large discrete hyaline droplets were present.

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In this individual, who died somewhat later in the course of the disease than the previous case, a definite increase in renal structural change is apparent. The subcortical congestion is more intense, though the excess blood is quite clearly contained within the dilated straight vessels of the outer medulla. There is also a marked congestion of glomerular capillaries and somewhat less of the

cortical intertubular network and both the large arteries and veins are greatly distended. One has the impression since there was no general visceral congestion observed post mortem to account for the excess blood in the kidney that dilatation of an atonic intrarenal vascular bed had by a reduction in peripheral resistance flooded the entire organ.

The other advance in the development of the renal lesion concerned the epithelium of the proximal convolutions. Throughout their length there was noted a frank protoplasmic disturbance.

* * * * *

Case 14 was a man admitted on the third day of his illness with a temperature of 105 and a blood pressure of 128/70 mm Hg on the third day in the hospital it fell suddenly to 90/74. The Three units of serum albumin were given. The urine showed a 3 + proteinuria on an intake of 800 cc his urinary output was 2280 cc the BUN was 24 mg per cent.

The renal lesion may be briefly summarized as similar to the preceding case except that the congestion was more closely limited to the subcortical medulla and the protoplasmic swelling of the cells of the proximal convolutions was more pronounced.

A further development of the disturbance in the cortex of the kidney was noted in Case 20. This man died of primary shock on the seventh day of his illness in spite of the repeated administration of infusions over a period of 3 days of serum albumin to a total amount of 8 units. There was no fluid intake other than by these infusions and there was a urinary output in 4 days of 1060 cc. His urine was negative for protein for the first 5 days then a trace appeared increasing in a few hours to 4 +. BUN on entrance was 22.9 mg per cent and on the day before death 85.0 mg per cent.

Histological examination showed the subcortical congestion and in the cells of the proximal convolutions the protoplasmic disturbance that has previously been described the latter had advanced to the formation of definite vacuoles. In addition irregular areas of edema were scattered through the cortex the cross sections of tubule being ... by collections of fluid (Figure ... al convolutions in the congested subcortical zone were filled with large hyaline droplets.

Case 26 was admitted on the third day of hi illness with a temperature of 105, his urine wa free of protein. Shock intervened almost im mediately and continued in spite of the a ministration of 16 units of serum albumin ove period of 3 days. On the fifth day of his illn the urine showed a 4 + proteinuria. His urir output during hospitalization was 460 cc. BUN rose to 69 mg per cent; he died in on the sixth day of the disease.

At autopsy massive retroperitoneal eder noted. The prosector was impressed dark appearance of the subcortical cor Histological examination showed this co in the form of dilated but intact intertub sels congestion was not excessive in th The glomeruli were normal but the capsu contained considerable amounts of p protein. The most pronounced a noted was the marked swelling of the thelium in the cortical portions of tl convolutions. Vacuolization was ext clear rounded spaces extending thr tire depth of the epithelial cells The terminal segments of proximal that lay in the subcortical zone of the bular vessels were less swollen the tained considerable amounts of cl protein in which were enclosed b and many desquamated cells w clei. The cells which still lined tubule were filled with hyaline d

The increasing alteration of the proximal convolution desc ceding three cases appeared at individual (Case C) who en with a temperature of 105.8 his illness went into shock morning. In spite of treat administration of 3 units of units of Dextran he succur edema on the eighth day. T cent on the first day of sho falling on the seventh day days the intake of fluid w nary output 1300 cc veloped and the BUN

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cent on the fourth day reaching 250 mg per cent on the day preceding death

At autopsy the kidneys showed a moderate degree of cortico-medullary congestion Retroperitoneal edema was present

On histological examination the usual dilation of the intertubular capillaries in the cortico-medullary zone was observed The most striking alteration was an extreme swelling of the epithelium of the proximal convolutions The finely granular cytoplasm of the cells appeared as if inflated, resulting in a protrusion of their apices into the lumen of the tubule, the brush border was, however, preserved, as were the nuclei which were displaced towards the base of the cells This intense swelling of tubular epithelium was sharply limited in its distribution to the proximal convolution, the epithelium of the ascending limbs being essentially normal (Figure 10) It extended into Bowman s space which was in part obliterated by the protrusion of the foamy cytoplasm of the cells of its parietal layer, the epithelium covering the tuft was not involved (Figure 11) No hyaline droplets were observed in the altered epithelium of the proximal convolutions

* * * * *

The evolution of the renal lesion as shown by the development of structural alterations in the preceding four cases is evidenced by two phenomena, increase in the intertubular congestion of the cortico-medullary zone and a swelling of the proximal convolutions The latter has the histological appearance of an increased hydration of the protoplasm of the epithelial cells which reached the point of vacuolization

The increase in subcortical congestion took the form of a spread in the extent of involved areas rather than in a progressive dilatation of individual channels In the less far advanced lesion, the groups of dilated intertubular vessels were widely spaced on a background of less affected medulla and had therefore the normal distribution of the horsetail-like strands of capillaries which result from the subdivision of each of the large, straight efferents from juxta-medullary glomeruli in the lower cortex (11) Originally forming isolated clusters, the addition of more dilated capillary radicles at their periphery resulted in their fusion to a more or less continuous band which in the end separated cortex from medulla The congested zone thus occupied the entire outer stripe and extended into the inner stripe of the outer zone of the medulla The altered vascular pattern is therefore that which might result from a continuing flow of blood into abnormal intertubular vessels that had lost their tonicity, the localized distinctiveness of this pattern will be considered later

Swelling of the tubular epithelium and protoplasmic disturbances in its cytoplasm have not received great attention in considerations of the pathogenesis of acute renal failure and are frequently dismissed under the stock description of "cloudy swelling" with the implication that the importance of this at times dubious histological lesion is not great Funck-Brentano has corrected this arbitrary view in his thesis on the physiopathological mechanisms of anuria in the acute nephropathies (12) In experiments on rabbits, oliguria and anuria were found to follow the infusion of serum containing 50 per cent glucose, whereas no such effect was produced by infusions of serum containing 9 9 per cent NaCl Sections of the anuric kidneys showed a marked swelling of the epithelial cells of the proximal convolutions very similar to the illustrations of the swollen epithelia in Figures 10 and 11 He concludes that the swelling is due to cellular hyperhydration which is derived as an effect of alterations in the osmotic pressure of the plasma, this he demonstrates by a corrected freezing point determination Similar changes in the freezing point of the plasma were noted in human clinical examples of the oliguric phase in acute renal failure There are no data available on plasma osmolarity in EHF, but the results of Funck-Brentano's experiments in the production of epithelial lesions by modification of osmotic factors and consequent shifts in hydration of cells are remarkably similar to those seen in EHF

In Case 20 of the preceding group there is noted for the first time in our description of the renal lesion the occurrence of hyaline droplets in the epithelial cells of the proximal convolutions, and these objects will be found in many examples that follow A consideration of their significance in this report would lead to a considerable digression We shall therefore only state that in our opinion these droplets do not represent an effect of the

disease processes though these do modify the conditions of their formation but rather are the result of a therapeutic procedure namely the administration of large amounts of human serum albumin. The hyaline droplets are in other words regarded not as the products of some hypothetical cellular 'degeneration but as droplets of absorbed modified protein similar to those which form in the cells of the proximal convolutions of experimental animals which have been given similarly large injections of homologous plasma proteins (13) Since the droplets are therefore not considered an essential feature of the renal lesion of EHF the data concerning them will be presented elsewhere in a general discussion of hyaline droplet formation in human renal disease and its significance in the metabolism of the plasma proteins

* * * * *

The three remaining cases from the Hypotensive Phase of EHF show increasing structural change of the nature so far described and the development of another aspect of the renal lesion that is to assume predominating proportions and result in grave renal damage in later stages of the disease namely the ill effects of the vascular disturbance on the tubules of the nephrons

Case A developed fever and nausea the day after his discharge from the hospital following an acute appendectomy from which he had apparently recovered with an uneventful convalescence Two days after readmission to the hospital a Douglas pouch abscess perforated and drained spontaneously he then went into shock An intra abdominal hemorrhage was suspected but none was found on laparotomy Transferred to the EHF hospital in shock he received 5 units of albumin and other treatment with little effect and died on the fifth day He was anuric during his last hospitalization his BUN rose to 64 mg per cent

At autopsy the kidneys presented the usual subcortical congestion and the anterior lobe of the pituitary was hemorrhagic.

Histological examination showed that in the renal cortex the epithelium of the convoluted tubules was greatly swollen and vacuolated resembling the alterations illustrated in Figure 9 there were few if any hyaline droplets in their epithelial cells except as the convolutions approached the cortico-medullary boundary In the area of subcortical congestion the excess blood was still contained within widely dilated capillary spaces The straight tubules in this region were filled with great numbers of what appeared at first glance to be hyaline casts these also extended upwards in the medullary rays (Figure 12) Collecting tubules in the deeper parts of the medulla and papillae showed only moderate numbers of casts

The terminal medullary segments of the proximal convolutions which lay in the congested subcortical zone were compressed and crowded together though their lumens were irregularly dilated and filled with hyaline material These collections on closer examination did not have the appearance of solid casts the great majority being composed of an albuminous fluid which had under the influence of the fixative coagulated in bubble like configurations containing debris and desquamated cells (Figure 13) The epithelial wall lying contiguous to the entrapped fluid was infiltrated with eosinophilic material its cells were disarranged in part desquamated and showed pyknotic nuclei The protoplasm of the better preserved cells was crowded with hyaline droplets of varying size.

Similar changes were noted in Case 21 who was admitted with a typical 3-day history of EHF and a temperature of 103° a blood pressure of 118/58 mm Hg and 1 + proteinuria After an uneventful 24 hours his hematocrit abruptly rose to 59 0 per cent and his blood pressure fell to 86/64 mm Hg with a pulse of 140 In spite of 5 units of serum albumin and other supportive treatment he died in primary shock on the next day the sixth day of the disease

At autopsy there was marked retroperitoneal edema the kidneys presented the typical appearance of subcortical congestion and there was a gross hemorrhage in the anterior pituitary

Histological examination showed the usual swelling and vacuolization of the proximal convolutions in the cortex and scattered areas of intertubular edema similar to those illustrated in Figure 8 In the markedly congested subcortical medullary zone the terminal proximal convolutions and ascending limbs were compressed and filled with hyaline material and desquamated cells

The intact epithelium of proximal convolutions was filled with hyaline droplets, these and the entrapped fluid are shown in the dissected specimens of Plate II Droplet formation was not limited to this region but extended throughout the entire length of the proximal convolutions up to the glomeruli, so that every cross section of them in the cortex was filled with large Gram positive droplets (Figure 14) The integrity of the cells, even when crowded almost to bursting was apparently preserved for, when visible among the massed droplets, their nuclei appeared normal (Figure 15)

Essentially similar alterations were observed in the kidneys of Case 8 This man was admitted with a typical 5-day history and a temperature of 103° On the sixth day he went into shock and in spite of treatment, including the administration of 4 units of albumin in 24 hours, he died on the next day His urinary output was 1410 cc , there was a 3 + proteinuria

At autopsy the usual subcortical congestion and pituitary hemorrhage were present The histological appearances were very similar to those described in the previous case hyaline droplets filling the cells in cross sections of proximal convolution throughout the cortex as well as in the terminal segments where entrapped albuminous material was concentrated

* * * * *

A resume may now be given of the development of the renal lesion as it is seen during the first week of EHF, a period including the Febrile and the Hypotensive Phases of the disease in individuals where its further evolution was arrested by death from circulatory collapse Combining the physiological, clinical and pathological data the following would appear to be the course of events

In the Febrile Phase which initiates the clinical syndrome the physiological evidence of high clearances indicates that renal blood flow is either normal or increased In the transition from the Febrile to the Hypotensive Phase, various clinical phenomena appear which indicate widespread damage to small vessels, capillaries and venules, the intense and increasing flush of the skin and mucous membranes and, more certainly widely dilated nail bed capillaries detectable by microscopic examination (14) are indications of loss of arteriolar tone and vasodilatation It is noteworthy that skin hemorrhages in the form of petechiae develop somewhat later A rising hematocrit without corresponding increase in serum protein concentration and the post-mortem finding of T-1824 which has escaped into massive retroperitoneal edema are conclusive demonstrations that plasma is now leaking from the damaged vessels The abrupt occurrence of a heavy proteinuria, which in the Febrile Phase had been insignificant or absent, shows that the renal capillaries as well are involved in this general vascular lesion

Renal blood flow now decreases as indicated by a depression of clearances (2, 3), this may occur independently of general circulatory collapse and is exaggerated when primary shock intervenes, as it did in all the cases of this series The result of this decreased flow through the kidney is, however, quite different in case of EHF than it is in the individual with normal renal vessels who suffers a similar circulatory depression as a result, say, of surgical shock In both instances there is one factor in common—the distinctive anatomical pattern of the vascular bed in the subcortical zone of the medulla, in the kidney of EHF, however, this peculiar vascular bed is abnormal since the vessels which compose it are both atonic and permeable

The vascularization of the medulla has been the object of extensive anatomical and functional study in recent years The older descriptions of the *arteria rectae* which supply it from efferents of glomeruli in the juxta-medullary zone of the cortex have been amplified by the studies of Trueta, Barclay, Daniel, Franklin, and Prichard (11) which demonstrate their relatively large diameter In extensive anatomical and physiological experiments, Block, Wakim, and Mann (15) have revealed the complexity of the reactions that occur in the subcortical zone to various abnormal situations, in particular those of shock and related conditions Barrie, Klebanoff and Cates have described this vascular bed most aptly as the "arcuate sponge" (16) and have pointed out the possibility of free communication by sinusoids between the arcuate arteries, the medullary network and the veins

Although there have been some differences in

interpretations of the functional effects of these anatomical peculiarities our examination of the disturbed renal circulation in the acute renal failure associated with traumatic and toxic injury (10) agrees in both its experimental and pathological aspects with the more detailed and exact studies of Block, Wakim and Mann (15) These investigators point out three constantly occurring phenomena in their varied experiments in which the renal circulation was disturbed by electrical stimulation of the renal nerves sudden clamping of the renal artery injection of substances such as epinephrine and the reduction by hemorrhage of the mean arterial pressure 1) when renal vasoconstriction operates in a renal vascular disturbance it appears to be limited to the cortex alone 2) under conditions of sharply decreased blood pressure and flow in the kidney the medulla may become congested even though blood is not flowing through it such an appearance therefore does not of necessity indicate an actual shunting of the renal circulation past the cortex through the medulla into the renal veins and 3) in all situations, as the blood flow *fails* it is best preserved in the cortico-medullary zone of the kidney They further point out that from the anatomical standpoint two paths are present for the flow of blood into the medulla—the long *arteriolae rectae* and the general network of peritubular capillaries which supply the cortico-medullary region When renal blood flow is reduced most of the flow from the cortex into the medulla is through the peritubular network into the cortico-medullary region with a corresponding decrease through the longer *arteriolae rectae* which pass to the deeper medulla Blood flow may therefore continue *into* but not *through* the cortico-medullary region under circumstances in which flow is reduced to a minimum elsewhere in the kidney

If in the light of these considerations one considers the situation in EHF in which after the increased influx of blood of the Febrile Phase a reduction of flow ensues in a renal vascular bed which is atonic dilated and permeable to the escape of plasma it is evident how and why the intertubular vessels of the medullary sponge become so intensely engorged with blood in spite of the decrease in RBF which characterizes the Hypotensive Phase As is illustrated in Figure 6 the contents of the subcortical capillaries become an almost solid mass of entrapped red blood cells Plasma which has escaped through the abnormally permeable vessel walls is found in areas of intertubular edema which may extend into the medulla (Figure 5) and by means of medullary rays into the cortex (Figure 8)

The effect of these circulatory disturbances on the nephrons is already apparent in the studies of individuals who died in primary shock In the earliest example (Case 38 Figures 1 and 2) they were so minimal as to be of doubtful significance as a factor in the renal lesion If death from circulatory collapse had not occurred it is reasonable to suppose that a return of renal blood flow would have resulted in complete restitution of a normal renal status In most of the examples of the Hypotensive Phase described however there was definite and increasing evidence of damage to nephrons so that here again the structural changes and the physiological aspects of the renal lesion correlate with the clinical indications of a beginning renal failure that were shown in the rise of BUN

The earliest tubular alterations to develop present indeed in slight degree in Case 38 were swelling and subsequent vacuolization of both the cortical and medullary portions of the proximal convolutions Except for the increase in volume of the renal cells and a decrease in the density of their cytoplasm which reached its maximum in Case C (Figures 10 and 11) there was no great alteration of their configuration, their nuclei appeared normal One might suppose, therefore that here again the renal lesion is reversible.

The similarity in appearance of the swollen epithelium of the Hypotensive Phase to the changes produced by Funck Brentano by experimentally altering the osmolarity of the plasma has been noted Whereas in his experiments hyperhydration of the epithelium may be considered the chief factor in the cause of oliguria acting either directly by luminal compression or indirectly through a modification of renal blood flow from increased intrarenal tension the relations are less simple in the case of EHF Decreased urine flow was irregularly observed during the Hypotensive Phase but not the definitive oliguria to be described later so that incidental factors such as variations in cardiac output fall in blood pressure, decreases in renal blood flow and consequent glomerular fil

tration would seem to be alternative explanations for the variation in renal output in the earlier phases of the disease

If the effects of cellular swelling and the pressure of engorged intertubular capillaries had not greatly interfered with urine flow through the lumen of the tubule, they nevertheless had produced their deleterious effect on the integrity of the nephrons This was apparent in the irregular compression, dilatation and entrapment of proteinaceous urine and desquamated epithelial cells in the terminal medullary segments of the proximal convolutions, as illustrated in Figure 13 and Plate II

In EHF, these tubular changes in the subcortical zone are the first evidences of a destructive damage to the nephrons which increases to irreversible and irreparable alterations in later stages of the renal lesion

THE RENAL LESION IN THE PERIOD OF TRANSITION

(The Incidence of Intertubular Hemorrhage)

The transition from the Hypotensive Phase to that of Oliguria is characterized by physiological and clinical phenomena which indicate that escaped plasma is returning to the circulation the hematocrit falls retroperitoneal edema decreases and direct measurements of plasma volume are higher In spite of these evidences of circulatory recovery fatal 'transition shock" may occur apparently due to arteriolar dysfunction (9, p 695) Eight fatalities occurring under these circumstances appear in this series, all of these showed the development of a structural alteration previously unencountered

This characteristic lesion of the Transition Period was the appearance of hemorrhage in the congested subcortical zone Whereas in the previous Hypotensive Phase the excess of blood was contained within greatly dilated intact intertubular vessels (Figures 4 and 6), foci of exudative hemorrhage were now seen scattered widespread through the area of congestion

Case 39 was admitted on the fourth day of his illness with a temperature of 101°, a blood pressure of 100/80 mm Hg and a 4 + proteinuria He went into shock shortly after admission and remained in circulatory collapse with remissions, in spite of 9 units of serum albumin and other treatment until his death on the seventh day On the fifth day, his hematocrit was 49 5 per cent His urinary output was 150 cc the day before death and the BUN was 50 mg per cent Certain details of his clinical course are shown in Text Figure 4

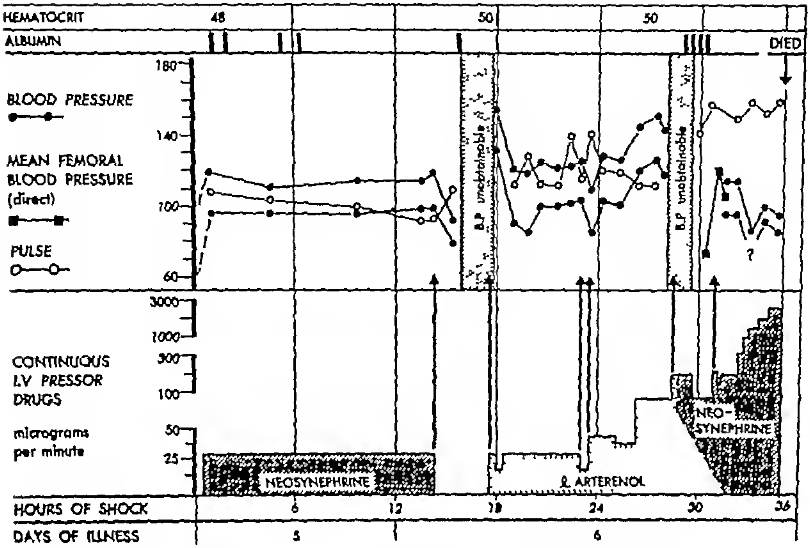
At autopsy there was only a moderate retroperitoneal edema, the kidneys showed the typical congestion of the cortico-medullary zone, and the usual extensive hemorrhage was present in the anterior pituitary

The general condition of the kidneys was an exaggeration of what has been observed in the more severe cases during the Hypotensive Phase Swelling and epithelial alteration of the proximal convolutions and scattered focal areas of intertubular edema were present in the cortex (Figure 16), entrapment of albuminous fluid and desquamated epithelial cells was seen in the straight tubules of the subcortical zone of congestion (Figure 17), which, along with irregular areas of intertubular edema, extended into the deeper medulla reaching the papillae The ducts of Bellini were filled with renal failure casts (Figure 18)

In the subcortical zone of congestion, many capillaries, though packed with red blood cells, were intact, though the outlines of the vessels were still visible, the intertubular spaces between certain tubules contained free red blood cells

Three other cases, 8, 4 and 27, were so similar in their clinical behavior and in the nature of the pathological lesions found at autopsy that a description of them would be needlessly reiterative All showed a somewhat higher elevation of BUN, on admission the BUN figures were 27, 49 and 12 mg per cent, respectively, and on the day before death, 117, 117 and 122 mg per cent Proteinuria showed a similar increase from 1 + on admission to 4 + on the day of death, which occurred on the ninth day of illness in the first case and the eighth day in the other two

Case 28, who died of transition shock on the tenth day illustrates the acme of intensity in the renal lesion present in this series He was admitted on the fifth day of his illness with many petechiae and a 3 + proteinuria He immediately went into shock In spite of temporary improvement under the usual treatment, which included the administration of 16 units of concentrated se-



TEXT FIG. 4 CASE 39—EXAMPLE OF TRANSITION SHOCK IN HEMORRHAGIC FEVER

Note the dependency of blood pressure on pressor therapy and the relative ineffectiveness of serum albumin therapy despite minimal increase in hematocrit.

rum albumin in 72 hours he passed from one episode of shock to another 14 in all to death on the tenth day The hematocrit was 53 5 per cent on the first day of hospitalization and 51 3 per cent on the day before death A urinary output of 200 cc. was noted on the first day and less than 50 cc. in the remaining period Blood urea nitrogen was 58 5 mg per cent on the day preceding death

At autopsy only a moderate retroperitoneal edema was observed, the kidney showed extreme subcortical congestion and there was a hemorrhage in the anterior pituitary

Histological examination showed the subcortical zone to be the seat of widespread infiltrative hemorrhage the intertubular capillaries which in examples of earlier stages of the disease were widely distended but whose endothelium was still intact were now no longer visible as distinct channels The fibrillar remnants of their walls however could be seen scattered through the irregular spaces which densely packed with red blood cells isolated each tubular cross section from its neighbor The enclosed tubules were compressed and distorted and their epithelium necrotic and desquamated so that the characteristic cellular pattern of the various tubular segments was lost (Figure 19)

This intense vascular disturbance separated the cortex by a broad band from the deeper medulla it was not however confined to the subcortical zone Extending into the cortex with decreasing intensity as the capsule was approached were extensions of the same extreme congestion and frank intertubular hemorrhage (Figure 20) In the areas free of congestion and hemorrhage the epithelial cells of the proximal convolutions showed the usual swelling and vacuolization of the earlier periods in tubules surrounded by infiltrating hemorrhage necrosis of portions of the entire wall including the basement membrane was apparent (Figure 21) The intact epithelial cells of the proximal convolutions were crowded with great numbers of hyaline droplets both in the

cortical convolutions and in those few terminal straight segments in the subcortical zone which were better preserved

Congestion, hemorrhage and tubular necrosis also extended deep through the medulla into the papillae Straight tubules, including collecting ducts, were thus isolated and compressed, their epithelium and even the basement membranes were necrotic over great stretches of their extent (Figure 22) The large ducts of Bellini were surrounded by widely dilated vascular spaces and scattered areas of hemorrhage, and contained renal failure casts (Figure 23)

Dissection of nephrons showed the nature and widespread distribution of the tubular damage, and in particular its topographic relation to the subcortical zone of intertubular hemorrhage

In Plate III A to D, all that remained of a proximal convolution is shown, the degree and extent of irregularly scattered tubular disruption as observed when a continuous tubule is examined, rather than in the interrupted cross sections of a histological section, are apparent Beginning at the origin of the convolution, its cellular pattern is markedly disturbed as can be appreciated by comparison with the more normal convolution of Plate I, nuclei are obscured in the disintegrating cytoplasm of the tubular epithelium, which forms irregular masses of deeply stained material alternating with areas of more lightly stained disrupted tubular wall The lesion increases in intensity (Plate III, B and C) as the straight segment leaves the cortex and enters the subcortical zone

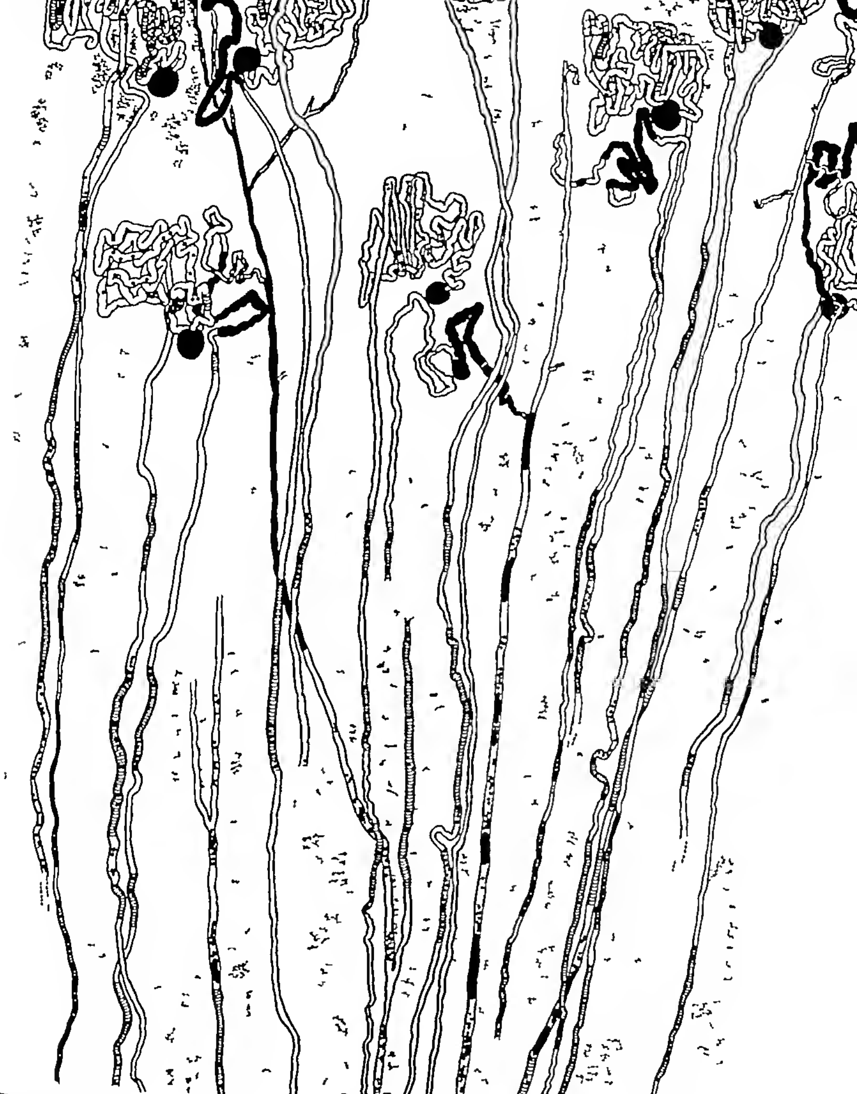
Here the tubule thins to a bare basement membrane which ultimately disappears as its course is interrupted by complete disintegration (Plate III D) An exact determination of the percentage of proximal convolutions thus physically interrupted was impossible since the lesion consists of a gradual attenuation of the tubule in which a few fibrillar remnants indicative of its former course remain Such remnants are so fragile that they cannot be dissected and though in a sense they might be considered still existent, they certainly can have none of the functional attributes of a tubule even the mechanical effect of constituting a confining channel for fluid Regarded from this simplest of tubular functions, only a few intact terminal proximal convolutions could be found in the zone of hemorrhage

As previously mentioned, the hemorrhagic area formed an almost continuous band between the cortex and deeper medulla, and extended to the papillae by irregular infiltrations Within these extensions of the hemorrhage all tubules showed in varying degree the deleterious effect of pressure and anoxia In Plate IV a fairly intact loop of Henle lying within the hemorrhagic zone is shown, throughout the course of which irregular stretches of damages are evidenced by cellular detritus and the obliteration of nuclear detail, at the bend of the loop the tubule is filled with a dense coagulum which stains black

Dissected collecting tubules passing through the zone of hemorrhage, though they had preserved their external configuration were in great part entirely necrotic, showing nothing of the distinctive clear cellular pattern which characterizes these structures, their luminal space was filled with debris and densely black stained casts (Plate V)

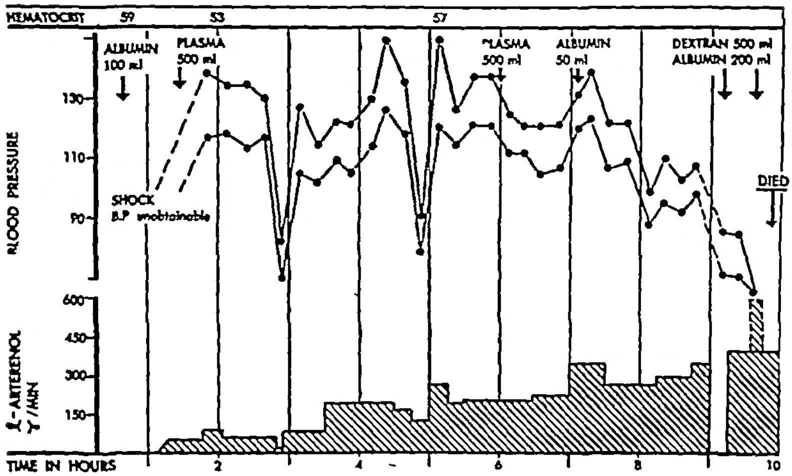
Returning to a description of the cortex in which lay portions of nephrons which in relation to the direction of urine flow, lay both before and beyond the interrupting band of subcortical hemorrhage, it was evident that the tubules distal to the zone were not only much better preserved than the severely damaged terminal proximal convolutions which lay in it, but in general were less severely affected than the first portions of the cortical proximal convolution which led directly from the glomeruli

In Plate VI, the origin of a collecting duct is shown with four ascending limbs which, passing into distal convolutions, join by way of connecting tubules to form the peripheral origin of the collecting system All these structures lay in the outer cortex beneath the capsule and were not involved by the irregularly scattered hemorrhagic infiltration that extended from the subcortical zone It will be noted that all these tubules are, compared to the severe damage illustrated in the cortical portions of proximal convolutions (Plate III), relatively well preserved There is, therefore, as in all examples of acute tubular necrosis and renal failure, no preferential damage to the "lower nephron" but rather the reverse The normal cellular pattern of the ascending limbs that lie in the cortex is visible although some are dilated and contain coagulated material and debris



TEXT FIG. 5 A RENAL LOBULE FROM CASE 28 WHO DIED OF TRANSITION SHOCK ON THE TENTH DAY

Note the extensive subcortical intertubular hemorrhage with extension into the cortex. Necrosis of tubules within the involved areas Heavy black glomeruli and casts within tubules cross-hatching necrosis of tubules small circles within tubules desquamated cells and debris Many terminal proximal convolutions end by disruption in the zone of hemorrhage. Magnification 18 ×



TEXT FIG 6 CASE 42—EXAMPLE OF FATALITY IN SEVERE PRIMARY SHOCK IN HEMORRHAGIC FEVER DESPITE CONTINUOUS L-ARTERENOL INFUSION

This patient was in severe shock when admitted and blood pressure was unobtainable until given both plasma volume expander and an infusion containing a large amount of L-arterenol Accidental stopping or slowing of the infusion resulted in marked decrease in blood pressure The severe shock that followed the second episode did not respond to further plasma volume expanders or heroic pressor therapy

The distal convolutions are also dilated, and though cellular detail is obscured by the intense black staining of the solid coagulum that distends their lumens, occasionally a flattened but intact epithelium is visible The connecting tubules are not dilated and in most instances are free of black stained coagula, this dense material appears again in the peripheral collecting tubules and, obscuring cellular detail, fills the remaining portion of the collecting duct

Text Figure 5 shows a reconstruction of a lobule from camera lucida drawings of dissected nephrons, it may be compared with Text Figure 3, a similar reconstruction of the earlier stage which showed so little departure from normal topographical relations The important features now noted are 1) the presence of an irregular band of hemorrhage separating cortex from medulla, 2) the extension of the congestion and hemorrhage to the papillae on the one hand and, in diminishing scattered areas, to the surface of the kidney on the other, 3) the interruption by necrosis of the majority of all the tubules, terminal medullary segments of proximal convolutions descending and ascending limbs—both broad and thin—and collecting tubules, which pass through the zone of hemorrhage, and 4) the dilatation with coagulated proteinaceous material of those tubules which lie in the cortex distal to the zone of hemorrhage

The clinical course of Case 42 is shown in Text Figure 6, admitted in a state of shock on the fifth day of illness, he died in 10 hours in spite of treatment that included the administration of 3½ units of serum albumin and 500 cc Dextran, 400 cc of urine were passed At autopsy a moderate retroperitoneal edema was present as well as the usual subcortical congestion in the kidney and hemorrhage in the pituitary

Histological examination showed an intense hemorrhagic infiltration throughout the corticomedullary zone The outlines of the intertubular capillaries could not be made out, red blood cells filling the spaces between the cross sections of tubules in densely packed masses Congestion and hemorrhage extended deep into the medulla and also into the cortex Many of the proximal convolutions in the cortex were filled with hyaline droplets, the nuclei of the cells containing them were well preserved In the areas of subcortical intertubular hemorrhage the tubules were compressed and distorted and their epithelial walls destroyed

The two remaining cases which showed massive intertubular hemorrhage in the congested cortico-medullary zone and which were considered clinically to have died in the late Hypotensive Phase or in the Transition Period of shock may be briefly mentioned, as the renal lesions were essentially similar to those which have been described Case 17 survived for 10 days the last 5 days of which he was in more or less continuous shock in spite of intensive treatment which included the administration of 12½ units of serum albumin during the last 4 days During this period he passed 190 cc of urine it is of interest that his urine had been free of protein during the Febrile Phase until the fifth day when with the onset of the Hypotensive Phase it suddenly showed a 4 + proteinuria

Case 1 was admitted on the fifth day of illness with a blood pressure of 90/60 mm Hg and died the next morning in shock Two units of serum albumin were given with a total fluid intake of 1500 cc his urinary output was 390 cc

The structural changes were essentially similar *in these two cases Intense subcortical intertubular* hemorrhage was present with extension into the papillae and less to the cortex with tubular destruction not only in the medullary zone but scattered irregularly throughout the cortex and deep medulla. In the individual who had received 12½ units of human serum albumin the cells of the better preserved convoluted tubules were filled with droplets these were present but sparse in the other who had been given 2 units

* * * * *

Since the period covered by the group of cases just described is by definition transitional from the Hypotensive to the Oliguric Phase a discussion of the structural findings can be postponed until the full development of the renal lesion in the next period has been given. An important correlation between clinical and pathological aspects should be noted in passing as the clinical phenomena required the special recognition of an interim between two major phases in the term Transition Period so the finding of a distinctive structural element intertubular hemorrhage sets this period off pathologically in the evolution of renal lesion. There has been no need for such a subdivision in descriptions of general Acute Renal Failure for no such structural distinction in the form of subcortical hemorrhage occurs

As will become apparent when the functional effects of this new development are described its importance in the ultimate fate of the kidney as a collocation of nephrons is critically determinative

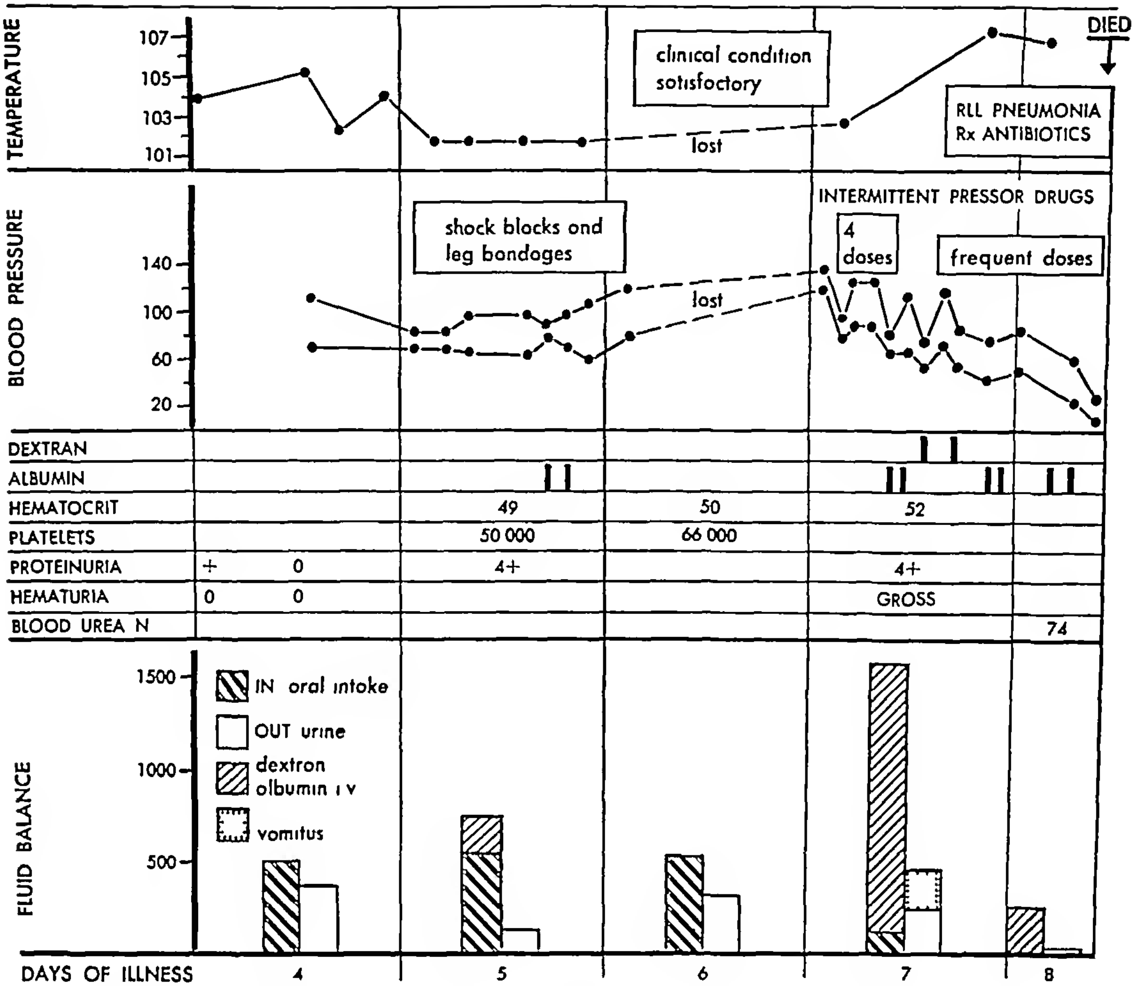
THE RENAL LESION IN THE PHASE OF ESTABLISHED OLIGURIA

(Infiltrative Intertubular Hemorrhage and Stasis Tubular Necrosis and Intrarenal Obstruction)

Intertubular hemorrhage resulting from increasing congestion that proceeds to ultimate stasis in damaged terminal vessels is not by the nature of its origin a sudden event but a gradual infiltration that develops at varying rates depending on the severity of both congestion and vascular damage It is not surprising therefore to find its structural and functional effects spread over three phases of the renal lesion the late Hypotensive the Transition Period and the Oliguric Phase

Ten cases each of whom died on or between the eighth to eleventh days presented the clinical characteristics of an established oliguria and showed the full development of the renal transformation of EHF The physiological disturbances in particular the detail of fluid balance, of a typical example Case 23 are shown in Text Figure 7 All cases showed the lesions which have been noted above in our description of those who died in late primary or transition shock *i e* a subcortical zone of intertubular congestion and hemorrhage with extension of both to the deep medulla and in lesser degree to the cortex, with tubular disruption of those portions of nephrons which were included in the affected areas

All ten cases showed in addition however an added structural change which characterized the Phase of Established Oliguria pathologically namely a marked dilatation of the cortical tubules of the nephrons This is illustrated in Case 31 an individual who entered hospital on the fourth day of his illness went through the Hypotensive Phase with shock for which he received 2 units of albumin and passed into the Oliguric Phase in which he died on the eleventh day with a BUN of 184 mg per cent. His fluid intake during the last five



TEXT FIG 7 CASE 23—EXAMPLE OF DEATH IN SECONDARY SHOCK DURING OLIGURIC PHASE OF HEMORRHAGIC FEVER

Primary shock on fifth day during Hypotensive Phase was mild Subsequent slight increase of hematocrit was presumably due to dehydration rather than to continued capillary leakage of plasma On the seventh day the patient developed right lower lobe pneumonia and hyperpyrexia that precipitated shock not responsive to therapy At autopsy characteristic findings of hemorrhagic fever were present, although there was no retroperitoneal edema In addition a patchy pneumonia chiefly in the right lower lobe was noted.

days was 4184 cc and his urinary output was 360 cc

Histological sections showed a wide dilatation of all tubules in the cortex In the case of the dilated proximal convolutions, the distention reached Bowman s space with a resulting compression of the glomerular tuft (Figure 24) Not only those portions of the nephrons lying proximal to the zone of medullary hemorrhage were distended but the cortical ascending limbs and distal convolutions the latter containing cellular debris, were also dilated The relation of this tubular dilatation to the compression of the subcortical zone of hemorrhage is shown in Figure 25

The effects of the dilatation of all the cortical portions of the nephron are shown in dissected specimens in Plate VII A to D The wide lumen of the proximal convolution is clearly apparent throughout its entire length, as can be seen by comparison with Plate I, the stretching of the epithelial wall increases the space between the nuclei, and so exaggerates the cellular pattern The epithelial cells are, however fairly well preserved As its terminal segment approaches and enters the

subcortical region of congestion it narrows to one third its previous diameter and is lost in the intertubular hemorrhage A portion of ascending limb and the distal convolution from the cortex which was originally entwined with the dilated proximal and which was therefore known to be the continuation of the same nephron show an equal dilatation there is some dark stained material in the distal convolution

In Case 25 there was more than the usual cellular damage in proximal convolutions and evidences of beginning repair were visible both in regenerative mitotic proliferation of epithelium (Figure 26) and in the ingrowth of interstitial granulation tissue into the disrupted tubule lumens Case 41 showed somewhat less dilatation of the lumens of the cortical tubules and scattered areas of intertubular edema (Figure 27) The remaining cases showed the general picture of cortico-medullary hemorrhage and dilatation of cortical tubules above it but added nothing further towards the elucidation of the renal lesion

* * * * *

It is in the Phase of Established Oliguria that the structural and functional lesions of EHF reach the height of their evolution When the structural changes in the nephron are thus fully established they appear to constitute an irreversible status for though a kidney of sorts might evolve from those nephrons which by the fortunate circumstance of their location had escaped the effects of congestion and hemorrhage the reconstitution of nephrons whose middle half has been destroyed would seem impossible We shall see in later descriptions that the attempt is made but the appropriate anastomosis of four frayed ends of fragile tubule lying loose in a mass of interstitial hemorrhage under considerable pressure is on the sheer basis of probability unlikely even when the potentialities of repair are present It would seem therefore, that the renal lesions we have seen in the fatal cases of established oliguria could not have existed in similar quantitative relations in the great majority of those suffering from EHF for they survived and went on to eventual recovery

In the earlier stages of the syndrome tubular damage was evident in the general swelling and vacuolization of the epithelium of the proximal convolution whereas in the Transition Period and Oliguric Phase the tubular alteration consists of localized physical disruption the two lesions are therefore quite different in their structural aspect Though the early epithelial changes are of the sort that have been classically derived from toxic action there has been no demonstration of a toxin in EHF other than the hypothetical substance which acts on the small blood vessels Disturbances of circulation however are clearly visible at this time so that it seems reasonable to accept them as the cause of the early epithelial damage

Structural interruption of the nephrons involves proximal convolutions ascending and descending limbs of Henle s loops and collecting tubules indiscriminately The damage is confined to the areas of congestion and hemorrhage, and so lies chiefly in the medulla but is also present extensively throughout the cortex when the circulatory disturbance has spread to this area Here again it would be difficult to explain this random distribution in terms of the direct action on the renal epithelium of a nephrotoxic poison derived hypothetically from the infectious agent of the disease The histological appearance of the epithelial lesion is also not that of nephrotoxic damage in which epithelial cells absorbing the poisonous substance die and desquamate within a tubule still maintained by an intact basement membrane There has occurred rather a disruption of the entire tubule wall the tubulorhexic lesion characteristic of the kidney of Acute Renal Failure (10) The inference seems certain therefore that the tubular lesions of EHF both early and late are the result of circulatory disturbances This conclusion does not of course rule out the primacy of an infectious agent or its products as the cause of the vascular disturbances for recent experiments by Thal (17) in the production of bilateral cortical necrosis by means of staphylococcus toxin show how this may be produced in the kidney and so result indirectly in extensive tubular damage There are many similarities between the nature of the tubular lesion in his experiments and what is seen in the kidney of EHF the differences in topographical distribution seem less important for as Thal points out cortical necrosis" is not so restricted as the descriptive term would suggest nor are the tubular lesions of EHF limited to the medulla

There is evidence that the circulatory disturbance acts on the tubule by means of two deleterious forces, the first, and perhaps less important, is physical pressure, and the second is anoxia following the congestive stasis and the infiltrative intertubular hemorrhage which has produced a cessation of circulation in the involved areas

Evidences of pressure, especially on the more sensitive proximal convolutions, were seen in the earliest stages of the renal lesion where there was only a simple and moderate congestion of the terminal vessels of the subcortical zone With an increase in vascular distention the effect of increasing intertubular pressure was evident in the compression and consequent entrapment of proteinaceous fluid in the lumens of the terminal proximals that lay in the involved area (Figure 13 and Plate II), and its final effect is demonstrated by the "intrarenal hydronephrotic" tubular dilatation which ultimately develops in the definitively oliguric kidneys (Figure 24) This dilatation involved all the tubules lying in the cortex above the subcortical zone of pressure and included not only proximal convolutions which are, in regard to the direction of urine flow proximal to the zone of compression in the medulla but also the distal convolutions, cortical ascending limbs and peripheral collecting tubules which lie distal to it

This apparent paradox of postobstructional dilatation arises from current misconceptions of the topographical arrangement in the human kidney of nephrons of varying length In most diagrams (Peter, 18, Figure 63) two types of nephrons are shown short-looped and long-looped, both of which pass through the outer stripe of the outer zone which in EHF is the zone of hemorrhage If this were an accurate and complete picture of the human kidney, the distal convolutions and collecting tubules would be cut off from the source of glomerular filtrate and could not dilate As a matter of fact the human kidney contains a very considerable number of nephrons similar to those shown in Peter's diagram of the pig's kidney (18) which turn within the cortex and never reach the medulla An example is given in our Plate VIII, A to C, many have been shown elsewhere (19) [4]

In a kidney with a zone of subcortical obstruction glomerular filtrate can therefore flow through uninterrupted cortical nephrons to the point of origin of the collecting system (*cf* Plate IXA) and from thence to all distal portions of the neighboring nephrons which are connected with it, even when they have been shut off from their normal source of filtrate by the zone of medullary hemorrhage This retrograde flow is in fact favored by the obstruction in the lower collecting system which itself passes through the obstructing zone of hemorrhage

The question may be asked why then do not all kidneys which suffer extensive disruption of their nephrons, such as, for example, Case 28 of the Transition Period, show extensive tubular dilatation in their cortices The answer would seem to be that for dilatation to occur there must be an adequate source of filtrate, a comparison of Figures 20 and 24 suggests that in the former case the extreme cortical congestion and hemorrhage is incompatible with a sufficient glomerular circulation and consequent formation of filtrate Thus a lesion which interrupts tubules in the medulla yet spares the circulation in glomeruli is necessary for the development of the tubular pattern observed in the Phase of Established Oliguria

The discussion of these minutiae concerning fluid flow in damaged nephrons would seem to warrant attention not so much for an importance inherent in their functional effect—for in both the situations described above urinary output was similarly lessened—but as illustrating how the same or similar pathological processes can by variation in their temporal or spatial relations modify or even change the direction of the evolution of the renal lesion In the examples cited it was a spatial relation, in the ensuing Phase of Diuresis it is temporal In any case, the simple statement that this lesion or that is present in a section of kidney is meaningless as a description of the structural-functional renal status

The structural evidence of physical impedance to the flow of urine through the nephrons is of

[4] Work in progress in this laboratory on quantitative aspects of the morphology of nephrons shows that their classical subdivision in the human kidney into two arbitrary groups of long-looped and short-looped nephrons is a considerable and, as in the present case at times misleading oversimplification Our findings show that the loops of nephrons are indeed of varying length but that this variation can be described by a normal frequency distribution curve rather than by absolute categories in short, some loops are longer than others

interest to others in addition to the theoretical pathologist The clinical observers who noted varying degrees of reduction in urinary output in the earlier Hypotensive Phase recognized the distinctive and definitive character of the oliguria which ensued in the later period and so designated it to the Phase of Established Oliguria The structural findings show that the distinction is real since it is based on a difference in causal mechanisms In the earlier Hypotensive Phase no structural lesion other than evidences of disturbed circulation was present in the kidney to account for decreases in urinary volume and it is therefore reasonable to suppose that factors such as variations in tissue hydration, cardiac output renal blood flow and glomerular filtration were responsible for this variable oliguria In the later phase what established the oliguria is clearly evident in the compression and destruction of tubules and the obstructional dilatation of nephrons that followed.

Of greater importance than pressure effects in the production of tubular damage is the anoxia that results from the stagnation and cessation of blood flow in the widely dilated and abnormally permeable intertubular capillaries of the subcortical zone It would appear likely that the *blood is moving with some difficulty under such* circumstances as are shown in an earlier period in Figure 6 where crowded red blood cells are packed to a solid hyaline mass Any doubts that circulation may cease entirely under these conditions have been answered by the conclusive experimental demonstration of Thal (17) who injected India ink into the general circulation of the living rabbit with an analogous renal stagnation hyperemia and found that it did not penetrate into vascular channels which were similarly distended and packed with red blood cells The further evolution of the congestive lesion in EHF through diapedesis to frank, diffuse intertubular hemorrhage ends therefore, in stasis

Under the conditions here described we are dealing with what Ricker in his classical study of the nature and effects of local circulatory disturbances (20 p 96 *et seq*) would have called rote stasis, and from the late irreversible stage of this rubrostasis he would have derived the Sequestrationsnekrose that interrupted the course of the nephrons It is of particular interest in our problem that in his examination of these effects in various tissues and organs he chose in the case of the kidney the necrosis that is produced experimentally by injection of vinylamin This toxic agent differs from other renal poisons by producing necrosis of tubules in the medulla rather than in the proximal convolutions of the cortex

Ricker points out that the lesion of vinylamin poisoning begins in the papilla with a prastatisch hyperemia followed by diapedesis of red blood cells, and progresses to Stasis" and eventual Dauerstasis The vascular disturbance in his experiments extended to the subcortical zone and in one instance to the cortex with resulting necrosis of tubules in the involved areas His conclusion that the tubular damage cannot be the result of either a functional secretion or absorption of the poison by epithelial cells as was suggested by Oka (21) or due to specific affinity for the poison as Levaditi *(22)* assumed has been convincingly confirmed by subsequent evidences that proximal convolutions Henle s loops and collecting tubules cannot be considered functionally similar hence it would not seem likely that they should all absorb a poison similarly or much less be given to the same affinities We can accept, therefore Ricker s explanation that the tubular necrosis is a phenomenon of ischemic sequestration due to the anoxia of the vascular disturbance [5] with his broader conclusion that local circulatory disturbances are mediated through the nervous system we need not here be concerned

THE RENAL LESION IN THE PHASE OF DIURESIS

(Intertubular Hemorrhage Tubular Disruption with Evidences of Resolution Regeneration and Repair)

The remaining cases in this series show structural changes that should be considered as illustrative of the effects of the passage of time on the

[5] Ricker also compares the action of vinylamin with that of mercury which damages the proximal convolutions this effect he also derives from circulatory disturbances and not from direct nephrotoxic action on the epithelium. Here, modern studies histochemical and pharmacological do not support his contentions, though it should be noted that he anticipated a later demonstration (10) that the vascular element of ischemia may play an important if subsidiary role in the production of tubular damage (tubulorhexis) in renal lesions of poisoning A recent study by Lindegård and Lofgren (23) has confirmed and extended these observations by demonstrating an irregular cortical ischemia not only in the anuric phase of mercuric poisoning but also in its diuretic phase.

fully developed lesions of the Oliguric Phase rather than as representative of the exact picture of the Diuretic Phase observed by the clinician during the recovery of the great majority of his cases If the individual in the Oliguric Phase does not die of secondary shock, dehydration, electrolyte imbalance or infection by the eighth to tenth days but survives another week or two the processes of resolution, regeneration and repair, as in all pathological complexes, begin their automatic operation A part of these mechanisms of restitution is the functional phenomenon of diuresis As the pathologist sees them, the progress of these processes, even if in the right direction, is blind and in the end futile, and the individual dies of a renal failure often obscured by some complication such as the infection of a bronchopneumonia, the hyperkalemia of electrolyte and fluid imbalance, or a cerebral accident It would seem unlikely that these complications can be merely incidental to the renal lesion, but rather it seems that in some manner, either directly or indirectly, renal insufficiency is the basis of their development, for in all the examples studied, though the complications were various the renal lesion with its grave structural damage and evidence of functional inadequacy in a BUN elevated at times to over 400 mg per cent was constant

What happens then in the great majority, 95 per cent of all cases of EHF has not been seen, the description of the typical kidney in the survivor must therefore be deduced and reconstructed on a hypothetical basis and will be further considered in our discussion

* * * * *

As happens not infrequently in the descriptions of passage from one phase of a disease to the next our first case, D, might well have been classified as an example of a renal lesion at the end of the Oliguric Phase, he survived, however, 15 days and as the urinary output, though continuing low, increased somewhat on the last day but one of his life the description was clinically placed under the title of the Diuretic Phase and will be here so considered As will be seen, there is no evidence of restitution of disrupted nephrons and in general the renal lesion resembles that observed in the previous phase

This patient was admitted on the fourth day of his illness with a typical history of the Febrile Phase The next day he abruptly developed a 4 + proteinuria From the seventh to the tenth day he was in the Hypotensive Phase with repeated shock that responded poorly to treatment, an established oliguria followed but the urine volume increased from around 15 cc in 24 hours to 250 cc on the day before his death On the twelfth day dialysis by the artificial kidney reduced the serum K from 6 9 to 4 2 mEq per L but the patient lapsed into coma, his temperature rose and coughing with bloody purulent sputum developed, a bronchopneumonia was confirmed by X-ray examination On the day before his death, the fifteenth day of illness, the BUN was 484 mg per cent

At autopsy the kidneys weighed 300 gm and showed the typical and fully developed lesion of subcortical congestive hemorrhage with deep medullary involvement There was a bilateral bronchopneumonia with multiple small abscesses containing Gram positive cocci, scattered hemorrhages in the right atrium gastrointestinal tract, the cerebral hemispheres and pons and the anterior pituitary

Histological examination showed that congestive hemorrhage was extreme in the subcortical zone and had extended irregularly and deeply into the medulla, reaching the papillae (Figure 28) In the outer zone there was an almost complete destruction by necrosis of all tubules contained in the areas of hemorrhage and these, coalescing, separated cortex from medulla (Figures 29 and 30) Extending downward into the infarct-like area of hemorrhagic infiltration could be seen wisp-like unoriented remnants of straight tubules, their walls were disrupted and in great part bare, but scattered along the denuded basement membranes were huge, atypical nuclei with intensely stained chromatin (Figure 31) Mitotic figures were frequent The intensity of this excessive regenerative proliferation distorted the tubular configuration so that it was impossible to accurately identify the parts of the nephron concerned in histological section

In the cortex the characteristic dilatation of the Phase of Established Oliguria was still present (Figure 32), involving Bowman's space and both proximal and distal convolutions, it will be recalled that in this patient's "diuresis" only 250

cc. per 24 hours was draining from the renal tubules The epithelium of the proximal convolutions was not only thin but cellularly atypical its nuclei were not round but oval and were irregularly distributed in the tubule wall in some places forming islands of excessive proliferation (Figure 33) the appearance was that of a regenerated epithelium Moreover the tubules were more widely spaced than normal (*cf* Figure 1) and the interstitial tissue was infiltrated with mononuclear cells and fibroblasts The glomerular tufts were large with widely dilated capillaries that contained discrete apparently circulating red blood cells

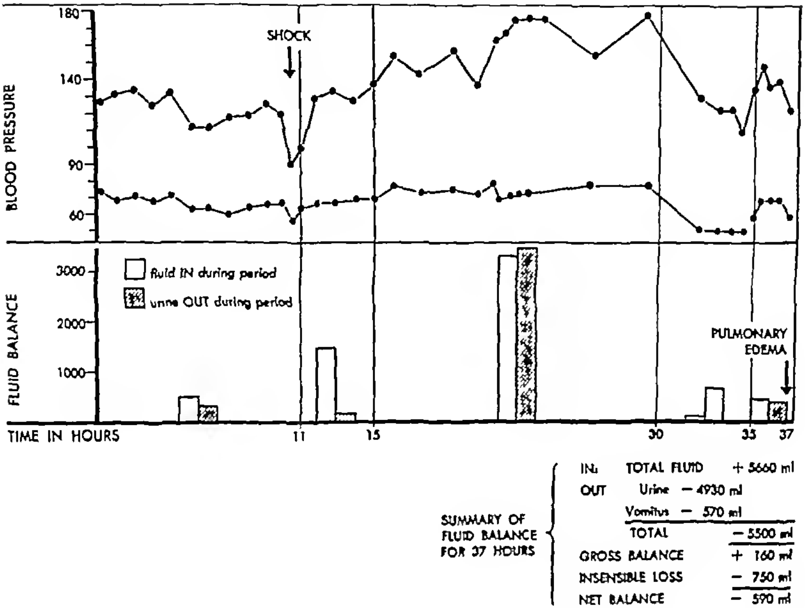
The topographical aspect of the lesions just described is shown in the dissected nephrons Plate VIII A to C shows a complete cortical nephron with a short loop that did not extend into the subcortical zone of intertubular hemorrhage and is therefore uninterrupted As in all examples of acute renal failure associated with traumatic and toxic injury the tubule is damaged from glomerulus to collecting tubule As is usual the damage is more severe in the proximal convolution than in the so-called lower nephron consisting of scattered areas of tubulorhexic disruption with the lumen containing desquamated epithelial cells Where disruption has not occurred the wall is composed of a heavily stained irregularly thin layer of atypical regenerated epithelial cells quite different from the plump evenly contoured cells of the normal tubule (*cf* Plate I) The transition to the loop of Henle is abrupt and there is as in all cortical nephrons only a suggestion of a thin portion From here on through the loop and ascending limb and distal convolution the tubule is greatly dilated with clear tubule fluid and contains desquamated cells and debris As is so often the case the short connecting tubule is less distended

Plate IX A to E shows the peripheral collecting system from its origin in a junction of connecting tubules of five nephrons that lay beneath the surface of the kidney to its destruction in the hemorrhagic zone of the subcortical medulla The connecting tubules of 9 nephrons in all filled with black stained material that obscures their epithelial pattern join to form a collecting tubule which is empty In many instances the collecting tubules contained similar material but this exceptional example was chosen for illustration because it shows the cellular pattern more clearly It will be observed that the epithelium is not disturbed until the proximity of the subcortical zone of hemorrhage is approached scattered particles of cellular debris then appear in increasing numbers until in the substance of the hemorrhagic zone, a complete necrosis and coagulation of the entire tubular wall is evident The tuning fork juncture with a neighboring collecting tubule which is similarly affected is well shown below this point the single common duct contains black stained material The appearance on histological section in other cases of similar completely necrotic collecting tubules which have nevertheless maintained their external configuration is shown in Figures 22 and 29 The general architectural pattern of the kidney is shown in Text Figure 8 in a reconstruction of the renal lobule composed of camera lucida tracings of dissected nephrons

In Case 43 a frank diuresis had been established for the last five days of the patient s life and the renal lesion has special interest because of all the examples of this phase of the disease that came to autopsy in this one alone resolution of whatever structural damage had occurred had reached at least the point at which one might imagine a chance of eventual functional restitution to be possible. The resulting kidney of such a problematical restitution would however have been severely deficient in its reserve' of nephrons

The patient entered hospital on the third day of his illness in the Febrile Phase with a temperature of 103 6 and on the fifth day went into typical shock of the Hypotensive Phase with the hematocrit reaching 66 per cent With appropriate treatment he passed through this episode to the Oliguric Phase, in which his average daily urinary output was approximately 100 cc. with a BUN rising to 280 mg per cent. His blood pressure also rose (170/106 mm Hg) and the hypervolemic syndrome accompanied by 5 convulsions followed Daily urinary output increased rather abruptly in 24 hours on the twelfth day from 190 to 1000 cc. and reached 4600 cc the day before his death During this terminal period he presented the difficult problem in water and electrolyte balance of a limited homeostasis illustrated in Text Figure 9 so that the margin between dehydration with the threat of secondary shock and ample hydration with impending pulmonary edema was represented

A Renal Lobule From Case D Who Died on the First Day of the Diuretic
…rrhage with destruction of tubules Heavy lined proximals …
… VIII A) other symbols as in Text Figure 5 M…



TEXT FIG. 9 CASE 43—EXAMPLE OF LIMITED HOMEOSTASIS DURING DIURETIC PHASE OF HEMORRHAGIC FEVER

These observations were made on fourteenth and fifteenth days of illness Death occurred on the sixteenth day 24 hours after study The patient was very dehydrated at the start of the observations having been in negative fluid balance for many days Note the response of shock and blood pressure to increased fluids between the eleventh and fifteenth hours. Also note decrease in blood pressure between the thirtieth and thirty fifth hours when output was allowed to exceed intake. Pulmonary edema occurred in last period when intravenous fluids were increased even though net balance was just barely positive during this time. (Note Right hand column, bottom line in 30 to 35 hour box should be cross hatched.)

by a net negative fluid balance of 600 cc During episodes of hypotension and shock the diuresis disappeared and urinary output was at oliguric levels to rise again to 4000 cc when arterial pressure was restored by nor adrenalin The last BUN taken the day before his death had decreased to 173 mg per cent and there was only a trace of protein in a urine of 1 011 specific gravity

At post mortem there was found a confluent bronchopneumonia hemorrhages in the right atrium cerebral hemispheres and basal ganglia, and in the anterior pituitary The gross appearance of the renal lesion is summarized in the autopsy protocol as lower nephron nephrosis slight

Histological examination showed a marked contrast to the pattern of a general dilatation of all cortical tubules seen in the previous stage of established oliguria the lumens of the proximal convolutions and other cortical tubules were not widely dilated but contained some granular material as did Bowman s spaces of glomeruli which were otherwise normal (Figure 34) The proximal convolutions were lined with an epithelium of the original normal type there was little evidence of any regenerative renewal of cells such as the irregularity in thickness and nuclear size and shape and staining affinity that characterized the epithelium of proximal convolutions in most cases at this stage of the disease The general appear

ance was therefore of a cortex which had never been greatly damaged

Contrasting, was the alteration in the subcortical outer stripe of medulla Extending downward from the cortex into this zone, which had the appearance of a region of resolution of hemorrhage and replacement fibrosis were the thickened terminal segments of proximal convolutions, their epithelium was quite atypical varied in thickness and containing many irregularly distributed large nuclei (Figure 35) In the broad band of the affected region irregular foci of typical hemorrhagic infiltration persisted alternating and blending with ill-defined areas where the tubules were separated not by masses of red blood cells but by cellular fibrous tissue (Figure 36), a considerable increase in collagenous fibrils surrounded atrophic tubules and fused with their thickened basement membranes (Figure 37) Extension of the fibrosis could be traced upwards into the lower cortex in the form of cellular scars which replaced tubules and surrounded glomeruli (Figure 38)

The renal lesions in five cases—two of which 16 and 46, died on the fourth day of diuresis two others 11 and 19, on the ninth day and one, 29, on the eleventh day—showed essentially similar lesions to those just described but with more evidence of previous damage and a lesser degree of restitution In the subcortical zone of the medulla, intertubular hemorrhage still persisted in all instances with intermingled areas of resolution and interstitial reaction (Figure 39) In two of the five cases 16 and 46 the epithelium of the cortical proximal convolutions was of the original mature type, in three, 11, 19 and 29, it resembled a replacement by atypical regenerated epithelium similar to that shown in Figure 40, in none were there the marked distention and dilatation of tubule lumens observed in the preceding Phase of Established Oliguria

The last two cases were of the longest duration that occurred in this series and therefore present the maximum effect of the passage of time on the renal lesion that was available for observation

The first of these Case 33, died on the nineteenth day of his disease in the tenth day of the Diuretic Phase He had passed through the Hypotensive Phase with two episodes of shock which responded to appropriate therapy and an Oliguric Phase of 4 days in which his urinary output averaged 250 cc in 24 hours when the BUN reached a maximum of 286 mg per cent During this period he was hypertensive (158/112 mm Hg) On the ninth day of his illness, diuresis began abruptly and continued until his death on the nineteenth day the urinary output ranging from 1410 to 4400 cc in 24 hours The last days of his life were an example of homeostatic instability with repeated episodes of dehydration hypotensive shock fluid replacement and threatening pulmonary edema During this terminal period he received 11 units of serum albumin Cerebral involvement became apparent, his temperature reached 104° and he died on the nineteenth day of his illness The BUN on the day preceding death had decreased to 145 mg per cent

At autopsy a confluent bronchopneumonia in addition to hemorrhages in the right auricle, pituitary, and interventricular septum were present The renal lesion is described as showing "a fairly marked degree of congestion and focal hemorrhage in the renal medulla"

In the histological sections the proximal convolutions in the cortex were lined with an atypical epithelium The lumens were therefore irregularly widened and contained a moderate amount of coagulated material and desquamated cells (Figure 40) The contrast in appearance between these tubules whose lumens were somewhat increased by the irregularity of their epithelium and the frank and even distention of a tubule dilated by internal pressure, as occurred in the Oliguric Phase, may be seen in a comparison of Figures 40 and 24, and of Plates X and VII Bowman's space was not dilated and the glomerular tufts appeared normal The tubules of the cortex were widely spaced and the interstices filled with a loose fibrous connective tissue in which were clusters of round cell infiltration In the subcortical zone of the medulla and extending to the papillae extensive areas of persisting intertubular hemorrhage were seen, in these areas the smaller tubules were widely separated and apparently reduced in number but the larger collecting tubules were not only intact but showed an extreme and irregular proliferation of their distinctive epithelium, which formed irregular plaque-like formations distorting the normally even contours

of their lumens (Figure 41) The resulting distortions of the terminal collecting tubules and the ducts of Bellini entering the pelvis the latter filled in part with renal failure casts are shown in Plate XI

Case 18 survived 27 days he had passed through the typical Febrile and Hypotensive Phases of the disease with only moderate shock On the seventh day of his illness he entered a 6-day period of Established Oliguria with hypertension and a rising BUN that continued without remission until his death reaching a final figure of 329 mg per cent On the twelfth day his urinary output increased from 195 to 2175 cc in 24 hours and a moderate but irregular diuresis at times of 3000 cc continued to his death During this terminal period the usual problem of maintenance of fluid balance between dehydration and pulmonary edema was present with repeated episodes of secondary shock In this period he received no serum albumin.

At autopsy a marked hypostatic congestion and edema of the lungs were present with the usual hemorrhages in right auricle and pituitary The renal lesion is described as showing the classical medullary hemorrhage with some evidence of resorption.

Histological examination showed extensive intertubular hemorrhage in the subcortical region extending deep into the medulla The areas of hemorrhage were not continuous but formed an irregular zone in which regions of tubular collapse and dilatation alternated with definite fibrosis of the intertubular tissue The dilated tubules lying in the fibrous areas extended by medullary rays into the cortex and were either empty or filled with hyaline material Proximal convolutions in the cortex were irregularly dilated their epithelium was of a normal mature type The glomeruli save for coagulated material in Bowman s space were not remarkable.

* * * * *

All of the individuals surviving the phase of oliguria for periods varying from 1 to 16 days showed widespread evidence of the usual renal reparative processes of epithelial regeneration and replacement fibrosis the general dilatation of cortical tubules was not present. The structural repair concerned the kidney tissue rather than the nephrons for there had occurred no restitution of organ structure in those elements whose continuity had been destroyed In Case 43 the histological pattern of the cortex with its glomeruli and proximal convolutions appeared not greatly abnormal (Figure 34) and at least from the structural viewpoint had the appearance of a possible functional adequacy even though renal insufficiency was still evident in the BUN which had decreased from a maximum of 280 to 173 mg per cent Judging from the amount of scattered scar tissue and distorted tubules in both the subcortical zone and in the cortex a considerable number of nephrons must have been in part destroyed

* * * * *

Further evidence of the nature of the repair is found in an individual who having recovered from EHF died of causes other than renal insufficiency 149 days later

This patient was first admitted to hospital on the third day of his illness with the typical clinical picture of EHF On the next day with a blood pressure of 110/90 mm Hg a pulse of 112 and a hematocrit of 60 per cent he was given one unit of serum albumin from then on the blood pressure was stable On the sixth day the urinary output dropped to 25 cc. on the seventh it was 125 cc he then became hypertensive (150/100 170/100 mm. Hg) On the tenth day diuresis of 1700 cc ensued the BUN which had risen to 70 mg per cent fell to 43 on the twelfth day He was transferred to a hospital in the States where 79 days after the onset of his illness a test of his maximum concentration capacity showed a specific gravity of 1 010 During this stay in hospital the patient presented symptoms of adrenal insufficiency cardiac irregularity and occasional clonic seizures In one of these he died 149 days after the onset of the attack of EHF

At autopsy the anterior pituitary was found to be almost entirely destroyed by an old hemorrhagic infarction the adrenal weighed 6.2 gm. in histological section there was a thinning of the cortex with a marked lipoid depletion and areas of necrosis in the zona glomerulosa the medulla was well preserved. The other endocrine glands save for atrophy and maturation arrest of spermatogenesis in the testicle were not remarkable

The kidneys weighed 370 gm , the surface of one was described as smooth, the other as finely granular

Histological examination showed similar lesions in both kidneys, but the cellular scars to be described were more prominent in one The subcortical zone of the medulla, which from the history may be assumed to have been the seat of circulatory disturbances, showed an increase in intertubular fibrous tissue and a consequent wide spacing of the tubules passing through it The majority of these straight tubules appeared normal, but scattered through the denser areas of fibrosis were collecting tubules which showed a hyperplastic proliferation of their epithelium (Figure 42) The deeper parts of the medulla, including the papillae, were free of fibrosis and appeared normal Rather different from the acellular fibrous scarring were other more recently appearing wedge-shaped areas of monocytic infiltration and fibrous proliferation which extended from mid-medulla through the cortex to the surface of the kidney Within the confines of these scars remnants of atrophic tubules and glomeruli were visible (Figure 43)

* * * * *

In the kidneys of this individual who had recovered from the acute episode of EHF there are two types of fibrous scarring and tubular destruction The one, present in the subcortical zone, is composed of an acellular fibrosis, the other, extending from medulla to cortex, is relatively afibrous and is filled with an infiltration of inflammatory monocytic cells The former corresponds in location and in its fibrous components with that seen in the reparative process in cases of shorter duration The frankly inflammatory nature of the latter type of fibrosis seems to be either a new element in the pathological complex or a greatly exaggerated one, since in the cases formerly described cellular infiltration of an inflammatory reaction though occasionally present, was at a minimum In the present case the noncommittal diagnosis of sub-acute pyelonephritis is at least warranted, it is a matter of speculation what relation it bears to the original lesion of EHF which appears to have healed with a benign fibrosis and the destruction of a certain number of nephrons

RESUME

Concurrent with our description of the structural changes in the kidneys during the succeeding phases of EHF have run a discussion of their significance as factors in the renal status and a critical consideration of the pertinent literature What now follows is an uninterrupted recapitulation of the course of the disease, attempting a chronological synthesis in structural-functional correlations that will integrate the renal lesion into the clinical syndrome

During the first hours of the Febrile Phase the individual shows clinical evidence in the intense flush of the skin and visible mucous membranes of vasodilatation of peripheral small vessels The capillaries of the nail folds are widely dilated, the same is apparently true of the small vessels in the renal vascular bed, as ERBF is either normal or increased It can be assumed, therefore, that the kidney during this first stage is flooded with a rapid circulation of blood through vessels which are, save for vasodilatation, essentially normal Since only traces of protein are found in the urine, it would seem that the glomerular capillaries have not as yet suffered the characteristic and catastrophic lesion of the disease which is to develop in the next stage

This vascular disturbance is revealed in the succeeding Hypotensive Phase by the abrupt leakage of plasma from the capillaries, and results in a reduced circulating blood volume It is associated with decrease in arteriolar tone so that hypotension and shock are the result The structural-functional correlation at this point is clear, for at autopsy the escaped plasma is found in the retroperitoneal spaces

Lowered arterial pressure of shock is a sufficient cause of decreased renal blood flow, but there are also local disturbances in the renal vascular system Proteinuria abruptly increases, often in the course of a few hours, as evidence of leakage of plasma through glomerular capillaries that have undergone damage similar to that which is so widely spread throughout the tissues, the correlative structural aspect of the lesion is seen in the precipitate which fills Bowman's space and in the areas of intertubular edema, analogous to the retroperitoneal edema, that are found in medulla and cortex Clearances show a sharp reduction in

ERBF even in the absence of clinical shock if the latter concomitantly or subsequently occurs as it did in most of the fatal cases a summation of general and local effects operates to reduce renal blood flow

It is the failure of renal blood flow, previously either abundant or excessive in an engorged vascular bed the arterioles of which have lost their tone and whose capillaries and venules are permeable to plasma but not to red blood cells that is responsible for the origin of the characteristic renal lesion of the disease The distinctive localization of this circulatory disturbance is due to the anatomical and functional peculiarities of the vascular system of the kidney which have been previously described Even with a reduced blood flow the atonic and permeable vessels of the medullary sponge in the subcortical zone of the medulla a region shown to be the last to suffer ischemia are flooded and distended to the point of tubular compression At this stage the circulation in cortex and deep medulla is not greatly disturbed

The status of the renal circulation is now that which Ricker (20) would have called a "prestatic congestion for judging from histological appearances the blood within the intact dilated intertubular capillaries is circulating There is little structural or functional effect other than moderate pressure on the tubules in the region of subcortical congestion Urinary output has been irregularly and variably reduced and follows in the main alterations of blood pressure and consequent renal blood flow In clinical terms oliguria has not been established

In the Transition Period and Oliguric Phase clinical evidences accumulate of an intensification of the general damage to small vessels and the consequent escape of more than plasma Petechiae in the skin increase to a maximum on the fifth or sixth day ecchymoses or hematomata at the site of trivial trauma hematemesis melena and hemoptysis all appear toward the end of the Hypotensive Phase along with a reduction in platelets So in the subcortical zone of the medulla hemorrhage at first by diapedesis and in the end by capillary disruption infiltrates the renal parenchyma All tubules in the areas of involvement now lie widely separated in a mass of non-circulating red blood cells

The circulatory disturbance has now passed from Ricker s prestatic phase to that of rubrostasis and is accompanied by necrosis of those portions of the nephrons which lie in the anoxic areas of congestive stasis Thus interruption of the intrarenal passages which tends to isolate cortex from medulla is not complete however for it is formed by the coalescence of focal areas of congestive hemorrhage which arose from the peculiar horsetail configuration of the *arteriae rectae* of the lower glomerular efferents The tubules of many nephrons and of the collecting system may be preserved even in what appears in section to be a severely affected kidney The importance of intact channels through the area of destruction for the continuation of some flow of urine in the Oliguric Phase and for its increase in the Diuretic Phase which may follow is apparent

The clinical observation of the development of an established oliguria during this period correlates with the pathological finding of compressed and interrupted intrarenal channels Not only are the compression and disruption of tubules seen in the subcortical zone of intertubular hemorrhage but more conclusive, the effect of that obstruction is evident in the consequent dilatation of cortical tubules both proximal and distal that lie above it Such retrograde alterations in the course of urine flow through the kidney are made possible by the presence of intracortical nephrons which have escaped medullary disruption It is noteworthy that this distention disappears with the onset of diuresis for it is not present in the kidney of those who have passed through a similar period and died in the Diuretic Phase

Tubular destruction is not limited to the subcortical zone of congestion stasis in the *arteriae rectae* reaches the papillae and the tissue lesions of hemorrhage and necrosis follow Extension into the cortex is less obvious yet nephrons from severely damaged kidneys regularly showed the tubulorhexic disruptive lesions of anoxia throughout the proximal convolutions

Those who died in the Phase of Established Oliguria and in the following Phase of Diuresis showed such extensive damage to both nephrons and collecting system that any adequate structural restitution in their kidneys would seem to have been impossible. We have previously considered in detail the mechanisms of both struc

tural and functional recovery after the tubular necrosis of acute renal failure (24), it is unlikely, therefore, considering the broad extent of tubule that may be destroyed by the tubulorhexic lesion that the repair of the damaged nephrons in EHF is possible Healing as has been demonstrated in the case of acute renal necrosis, is due to formation of scars that contain afunctional remnants of destroyed nephrons

Survival of the individual and ultimate recovery therefore depends on the escape of an adequate number of nephrons from anoxic necrosis Since 95 per cent of the individuals with EHF survive and, in routine clinical examination during convalescence, appear to have recovered normal renal function, it follows that the great majority never could have developed the extensive structural damage that was present in those who died in oliguria or later diuresis In the typical case of EHF the turning point towards recovery or exitus comes in the Transition Period, if the vascular disturbance remains at the level of a prestatic congestion with little or only moderate intertubular hemorrhage and consequent destruction of a few nephrons, then a return of adequate blood flow is the major requisite for the restitution of the renal status These relations are illustrated graphically in the lowest subdivision of Text Figure 1 Individuals recover from the renal lesion of EHF not by repair of nephrons but because a great number of their nephrons have not been irreparably damaged

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frank epithelial structural lesions in the "stage of diuresis," and marked epithelial destruction and intertubular edema in the later "stage of oliguria" (28, 29)

In a tentative manner one might imagine analogous tubular disturbances operating in the complexity of the renal lesions of Acute Renal Failure and EHF Under clinical conditions the sequence of events in the two diseases is reversed, oliguria and structural damage of the grave renal insult preceding a phase of restitution of renal function that is indicated by diuresis when tubules, still inadequate, fail to absorb the fluid of an increasing glomerular filtration that has followed a return of renal circulation

As this discussion has progressed comparisons and analogies have continuously presented themselves relating the structural and functional aspects of the renal lesion of EHF to those of the renal lesion of Acute Renal Failure which is associated with all the various forms of traumatic and toxic injury In another place (30) these renal lesions have been compared, in their merging patterns that lie between two types of structural alteration, nephrotoxic and tubulorhexic, to the gradation of spectral bands that, individual, yet blend to a continuum, in this spectrum each case presents its characteristic signature which is derived from the peculiar qualities of its clinical origin On such a spectrum the distinctive elements of the renal lesion in EHF, such as its prominent Transition Period and the Established Oliguria Phase with its causal relation to the interruption of urinary channels by the characteristic subcortical zone of congestive hemorrhage, stand out in bright lined contrast, appearances both functional and structural, are very similar and yet sharply different from those of Acute Renal Failure It would seem possible to resolve this apparent anomaly and rationalize our metaphor by the recognition that the renal lesion in EHF is Acute Renal Failure in an individual whose peripheral vascular bed, including the renal, is atonic and permeable as a result of an infectious disease Thal's experiments (17) have removed all doubts that infectious noxa can produce the vascular disturbance of renal ischemia and its distinctive tubulorhexic necrosis

There still remains the task of establishing what the exact nature of the infectious noxa in EHF may be It has been shown that it does not have the characteristics of a histamine-like substance, and preliminary evidences of a specific vasodilator substance that circulates in the plasma have so far proved inconclusive (31) As in the more fundamental problem of the nature of the causative agent in the disease, it would seem that here lies hidden what might prove to be the definitive contribution to the understanding of the renal lesion in Epidemic Hemorrhagic Fever

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FIGURE 1

FIG 1 CASE 38, DIED ON FOURTH DAY OF PRIMARY SHOCK AT THE BEGINNING OF THE HYPOTENSIVE PHASE—THE OUTER CORTEX

Except for some swelling of the epithelial cells of proximal convolutions and dilatation of distal tubules, which appear empty, the renal elements are essentially normal Magnification 150 ×

FIGURE 2

FIG 2 JUNCTION OF LOWER CORTEX AND MEDULLA SHOWING DILATATION OF PROXIMAL CONVOLUTIONS ENTERING THE MEDULLA AND OF ASCENDING LIMBS PASSING TO THE CORTEX MAGNIFICATION 150 ×

FIGURE 3

FIG 3 MID PORTION OF OUTER STRIPE OF OUTER ZONE OF THE MEDULLA

In the central lower half can be seen the irregular and diffuse pattern of dilated intertubular capillaries crowded with red blood cells Magnification 150 ×

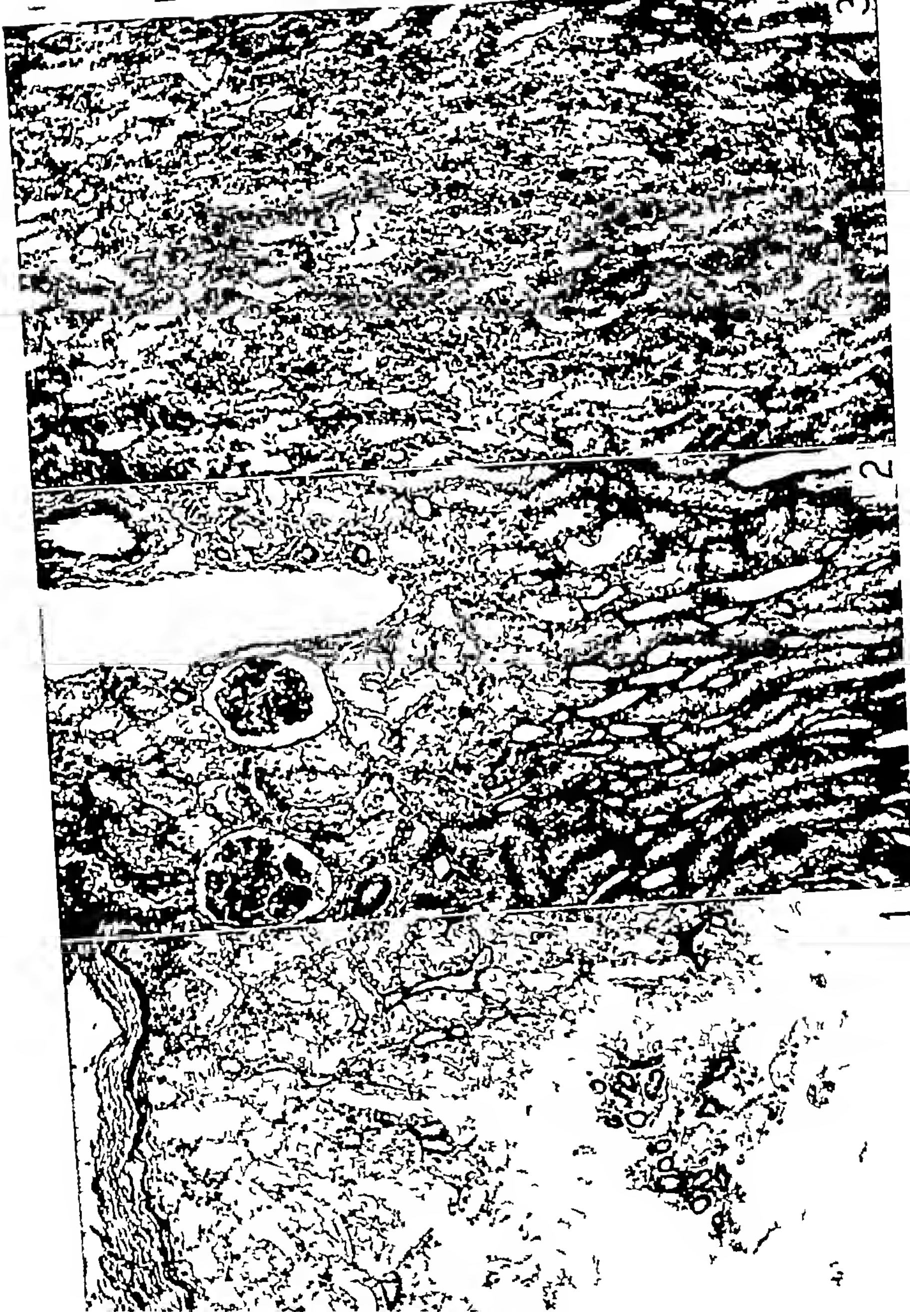
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FIGURE 4

FIG 4 THE CONGESTED AREA OF THE SAME KIDNEY AS SHOWN IN FIGURE 3

The thin endothelial walls of the intertubular capillaries are intact and their greatly dilated lumens are filled with discrete red cells which gives the appearance of circulating blood There is no hemorrhage Magnification 300 ×

FIGURE 5

FIG 5 THE DEEP MEDULLA

The collecting tubules are well preserved, there is no great congestion but there is extensive intertubular edema. Magnification 150 ×

FIGURE 6

FIG 6 CASE 9, DIED ON THE FIFTH DAY IN THE HYPOTENSIVE PHASE OF PRIMARY SHOCK—AN AREA OF SUBCORTICAL CONGESTION SIMILAR IN LOCATION TO FIGURE 4

The thin endothelial walls of the intertubular capillaries are intact, their distended lumens crowded with red cells which have fused to hyaline masses, thus giving the appearance of stagnation of flow or stasis Other cross sections are of tubules with flattened epithelium (thin loops?) containing hyaline casts Magnification 300 ×


4
5

FIGURE 7

FIG 7 MID-CORTICAL REGION SHOWING A GREATLY CONGESTED GLOMERULAR TUFT

There is considerable leakage of protein into Bowman's space In the two cross sections of proximal convolution, beginning swelling and vacuolization are apparent Magnification 500 ×

FIGURE 8

FIG 8 CASE 20, DIED OF PRIMARY SHOCK ON THE SEVENTH DAY IN THE HYPOTENSIVE PHASE—AN AREA OF CORTICAL INTERTUBULAR EDEMA SURROUNDING DISTORTED CROSS SECTIONS OF PROXIMAL CONVOLUTIONS THE EPITHELIUM OF WHICH SHOWS SCATTERED CLEAR VACUOLES—MAGNIFICATION 500 ×

FIGURE 9

FIG 9 CASE 26, DIED ON THE SIXTH DAY OF PRIMARY SHOCK IN THE HYPOTENSIVE PHASE—EXTREME SWELLING OF THE PROXIMAL CONVOLUTIONS THROUGHOUT THE CORTEX WITH MARKED VACUOLIZATION OF THE EPITHELIAL CELLS—MAGNIFICATION 500 ×

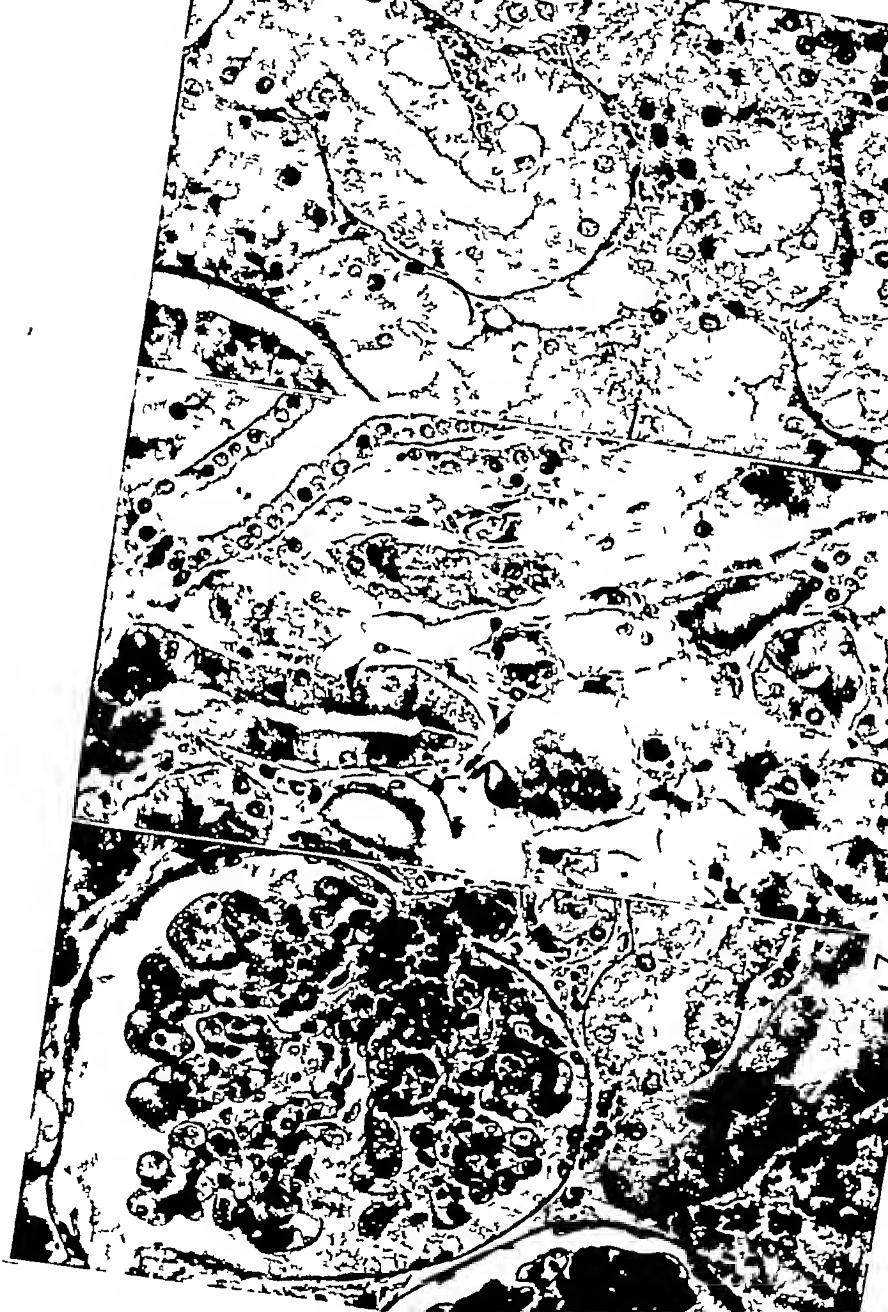

FIGURE 10

FIG 10 CASE C, DIED ON THE EIGHTH DAY WITH PULMONARY EDEMA IN THE HYPOTENSIVE PHASE—TRANSITION OF CORTEX TO MEDULLA SHOWING EXTREME FINE VACUOLIZATION OF PROTOPLASM OF CELLS OF TERMINAL SEGMENT OF A PROXIMAL CONVOLUTION

Note the well preserved brush borders Magnification 500 ×

FIGURE 11

FIG 11 VACUOLAR SWELLING OF TUBULAR EPITHELIUM IN CORTICAL PROXIMAL CONVOLUTION WITH EXTENSION INTO BOWMAN'S SPACE AND COMPRESSION OF GLOMERULAR TUFT—MAGNIFICATION 500 ×

FIGURE 12

FIG 12 CASE A, DIED ON THE FIFTH DAY OF PRIMARY SHOCK IN THE HYPOTENSIVE PHASE—A MEDULLARY RAY IS SHOWN PASSING HORIZONTALLY THROUGH THE MIDDLE HALF OF THE FIGURE

The *majority of the* tubules (ascending limbs of Henle's loop) are filled with clear hyaline casts Magnification 150 ×

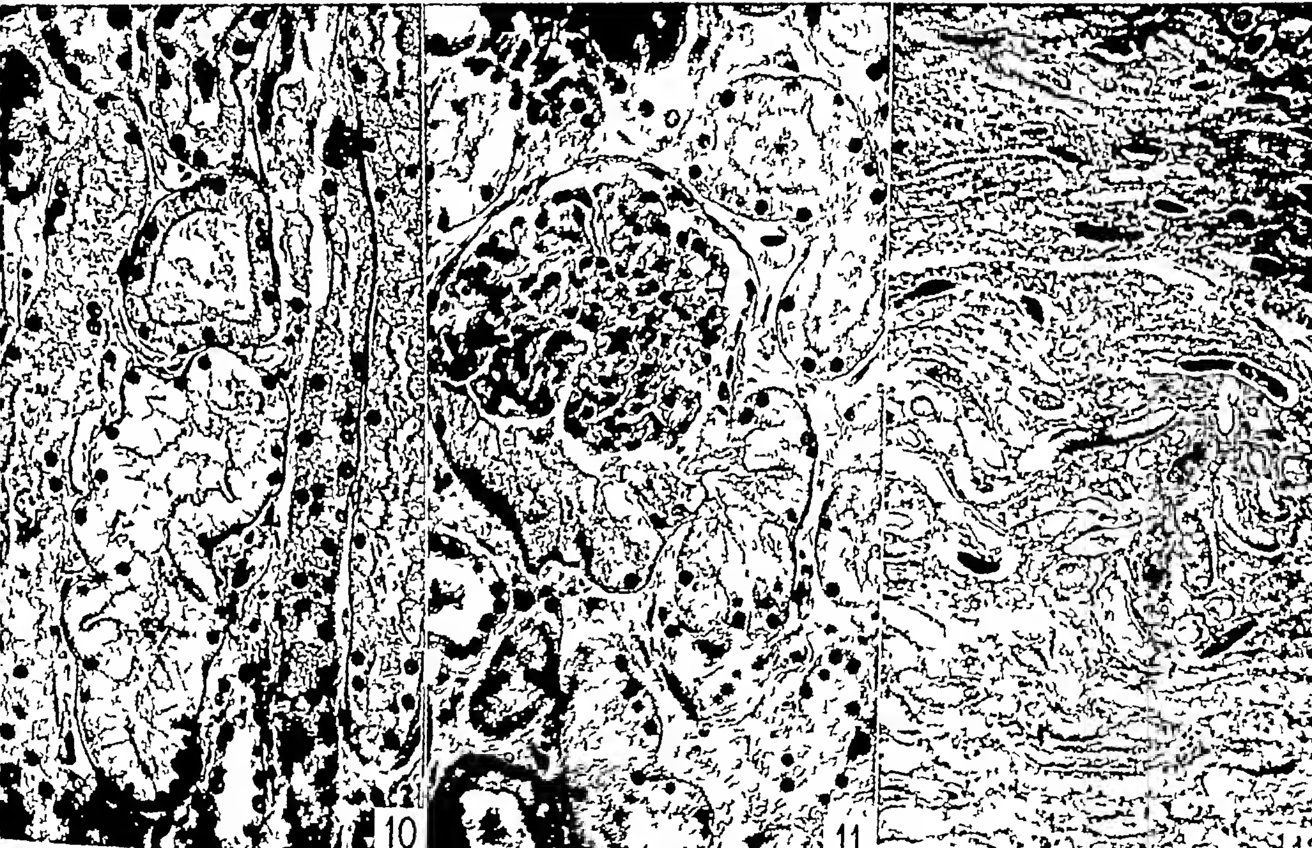
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Figure 13

Fig 13 Terminal Segments of Proximal Convolutions Lying in the Subcortical Zone of Congestion Showing Irregular Compression and Distention of Their Lumens Which Contain Coagulated Proteinaceous Material

The lining epithelium is either flattened or filled with hyaline droplets Magnification 500 ×

Figure 14

Fig 14 Case 21, Died on Sixth Day of Primary Shock in the Hypotensive Phase—He Received 5 Units of Human Serum Albumin in the 36 Hours Preceding His Death

Section of cortex, stained with Gram, showing practically *all cross sections* of proximal convolutions filled with Gram positive droplets Magnification 100 ×

Figure 15

Fig 15 Same Preparation at Higher Power

Every cross section of proximal convolution in the field is crowded and distended with Gram positive droplets The nuclei of the epithelial cells, when visible, are well preserved Magnification 500 ×

13
14

FIGURE 16

FIG 16 CASE 39, DIED ON THE SEVENTH DAY OF SHOCK IN THE TRANSITION FROM THE HYPOTENSIVE TO THE OLIGURIC PHASE—ONE OF THE SCATTERED AREAS OF INTERTUBULAR EDEMA IN CORTEX

The epithelium of the proximal convolutions is swollen, a distal convolution contains a solid cast Magnification 500 ×

FIGURE 17

FIG 17 ENTRAPMENT OF PROTEINACEOUS MATERIAL CONTAINING MANY DESQUAMATED EPITHELIAL CELLS IN "STRAIGHT TUBULES" LYING IN THE SUBCORTICAL ZONE OF CONGESTION

The intertubular capillaries are distended with red cells Magnification 500 ×

FIGURE 18

FIG 18 THE PAPILLA, SHOWING EXTENSIVE EDEMA AND MARKED CONGESTION WITH BEGINNING HEMORRHAGE

The large ducts of Bellini contain the renal failure casts of Addis Magnification 150 ×

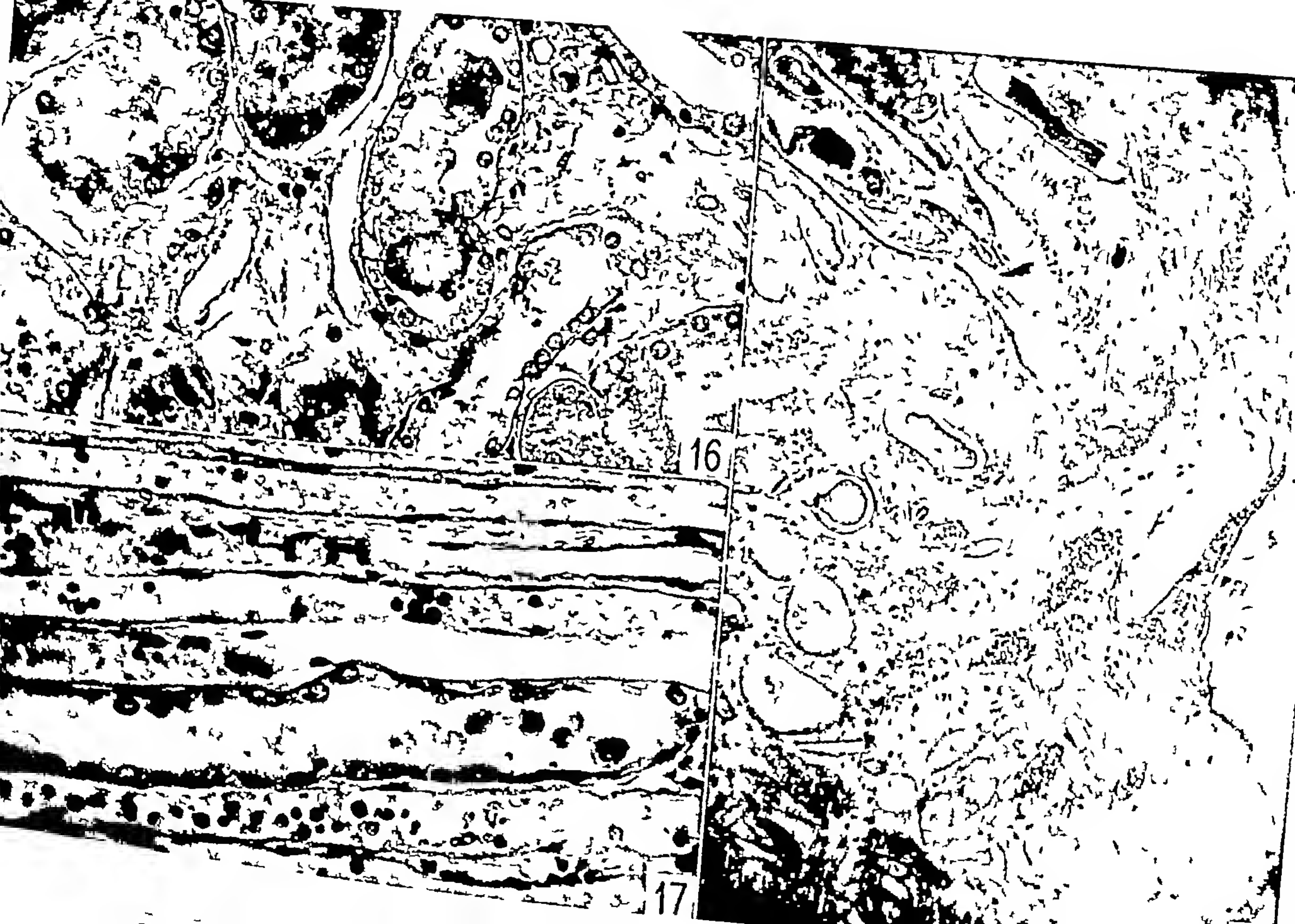
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FIGURE 19

FIG 19 CASE 28, DIED ON THE TENTH DAY OF TRANSITION SHOCK—THE SUBCORTICAL ZONE OF HEMORRHAGE

No remnants of the intertubular capillaries remain in a mass of crowded red blood cells which isolates and compresses enclosed degenerating and necrotic tubules, above, the remnant of a terminal segment of a proximal convolution, below, less damaged collecting tubules Magnification 500 ×

FIGURE 20

FIG 20 THE CORTEX, SHOWING IRREGULAR EXTENSION OF THE INTERTUBULAR HEMORRHAGE FROM THE SUBCORTICAL MEDULLA

Proximal convolutions are surrounded and widely separated by the infiltration which reaches in places to the capsule *Magnification 150 ×*

FIGURE 21

FIG 21 DETAIL OF CORTICAL INTERTUBULAR HEMORRHAGE AND ITS EFFECT ON THE ENCLOSED TUBULES

In the left center three cross sections show the typical appearance of the tubulorhexic lesion in histological section, in each, a portion of the entire wall is destroyed and yet the remaining cells and basement membrane are remarkably well preserved and intact Magnification 500 ×

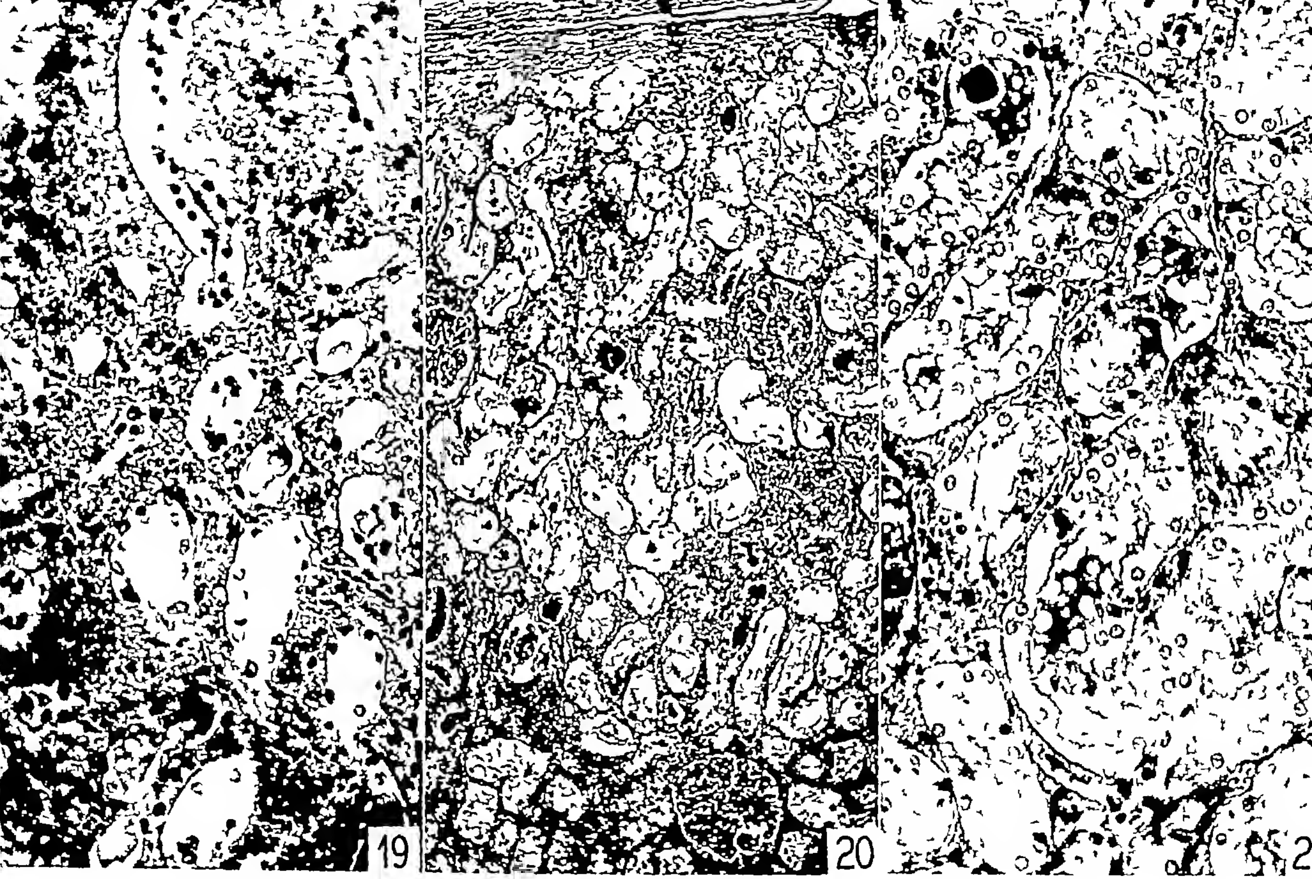
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FIGURE 22

FIG 22 MID MEDULLA, SHOWING COMPLETE NECROSIS OF ALL TISSUES INCLUDING TUBULES, MOSTLY COLLECTING, WHICH STILL MAINTAIN THEIR EXTERNAL CONFIGURATION

To the right, stasis in engorged vessels crowded with compacted red cells, there is no extensive hemorrhage Magnification 150 ×

FIGURE 23

FIG 23 THE PAPILLA—EXTENSIVE CONGESTION WITH STASIS AND HEMORRHAGE

The necrotic ducts of Bellini are still recognizable and are filled with huge renal failure casts Magnification 150 ×

FIGURE 24

FIG 24 CASE 31, DIED ON THE ELEVENTH DAY IN THE OLIGURIC PHASE—THE TYPICAL APPEARANCE OF THE CORTEX IN THE STATE OF ESTABLISHED OLIGURIA

Dilatation of all urinary channels above the compression of the zone of intertubular hemorrhage Bowman's space, proximal convolution and distal convolution (two cross sections contain solid debris) are greatly and evenly distended with resulting pressure effects on tufts and epithelium Magnification 150 ×

22

23

Figure 25

Fig 25 Outer Stripe of the Outer Zone of the Medulla

Below, the intertubular hemorrhage is so extreme as to obliterate tubules, above, in the transition to the cortex is seen the beginning dilatation that extends into the cortical tubules shown in Figure 24 Magnification 150 ×

Figure 26

Fig 26 Case 25, Died on the Tenth Day in the Oliguric Phase

The large cross section of proximal convolution shows an irregular *but* living epithelium in which are two mitotic figures Immediately above, in a smaller cross section one-half of the tubular wall is composed of atypical regenerated epithelium, the other of invading intertubular connective tissues Magnification 500 ×

Figure 27

Fig 27 Case 41, Died on the Tenth Day in the Oliguric Phase—One of the Scattered Areas of Cortical Intertubular Edema

The deforming effect of external pressure on the irregular dilatation of proximal convolutions is apparent Magnification 500 ×

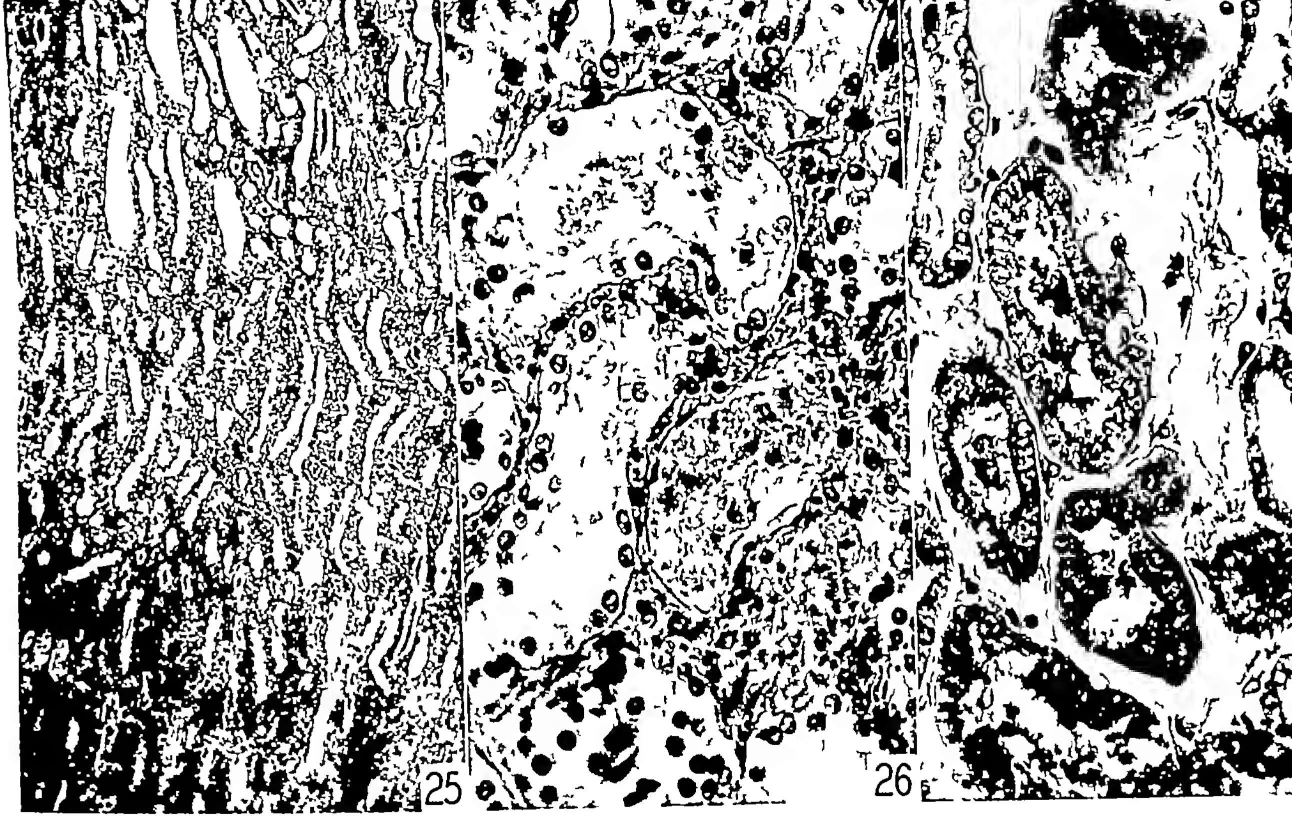

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FIGURE 31

FIG 31 SOMEWHAT DEEPER IN THE MEDULLA

In the upper left, necrotic terminal segments of proximal convolutions, below, wisp like terminal segments with huge hyperchromatic giant cell nuclei extend into the region of intertubular hemorrhage Magnification 500 ×

FIGURE 32

FIG 32 CORTEX SHOWING THE PERSISTING OLIGURIC DILATATION OF CONVOLUTIONS, PROXIMAL AND DISTAL, AND OF BOWMAN'S SPACE—MAGNIFICATION 150 ×

FIGURE 33

FIG 33 HIGHER POWER VIEW OF SAME DILATED CORTICAL PROXIMAL CONVOLUTIONS

The epithelium shows the oval and irregular nuclei of flattened regenerated cells which have replaced the original epithelium which had been destroyed by necrosis and desquamation Magnification 500 ×

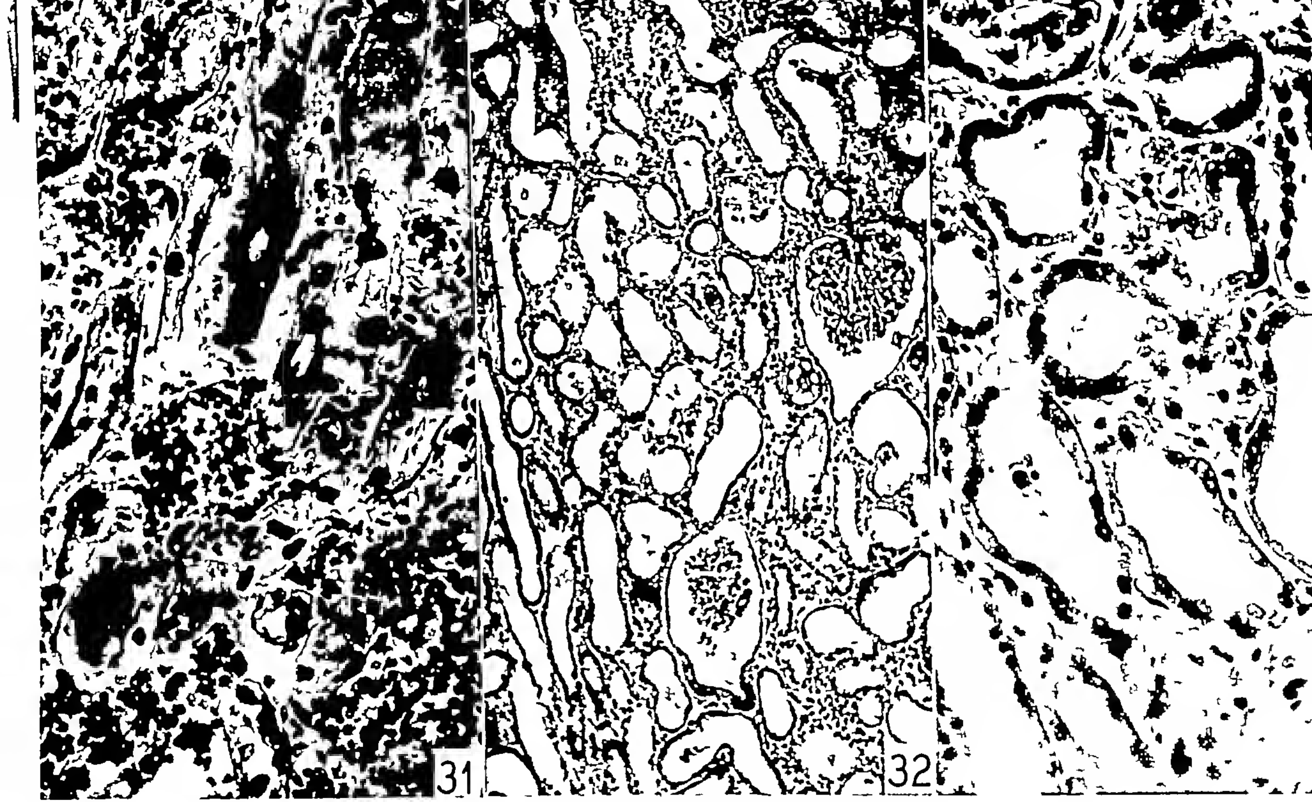

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FIGURE 34

FIG 34 CASE 43, DIED ON THE SIXTEENTH DAY OF HIS ILLNESS AND IN THE FIFTH DAY OF THE DIURETIC PHASE—OUTER CORTEX, CAPSULAR SURFACE TO LEFT

The proximal convolutions are lined with epithelium of their original normal type and the dilatation of the Oliguric Phase has disappeared The appearance suggests that not much tubular damage had occurred at any previous phase of the disease. Magnification 150 ×

FIGURE 35

FIG 35 OUTER STRIPE OF OUTER ZONE OF MEDULLA

Extending down from the cortex are the terminal segments of proximal convolutions They are lined with a deeply staining atypical regenerated epithelium The destruction of the original cells of these tubules was the result of the intertubular hemorrhage in this region (*cf* Figure 30), this hemorrhage is no longer apparent and the intertubular spaces are filled with fibro blastic tissue. Magnification 350 ×

FIGURE 36

FIG 36 A DEEPER LEVEL IN THE MEDULLA

Among areas of persisting intertubular hemorrhage ill defined patches of resolution are seen where, between atypical hyperplastic tubules, mostly collecting, a fibroblastic connective tissue has replaced earlier hemorrhage Magnification 150 ×

FIGURE 37

FIG 37 OUTER MEDULLA

The Masson stain shows the increase in fine collagenous fibrils between tubules in an area of resolution with thickening of the basement membranes of tubules Magnification 350 ×

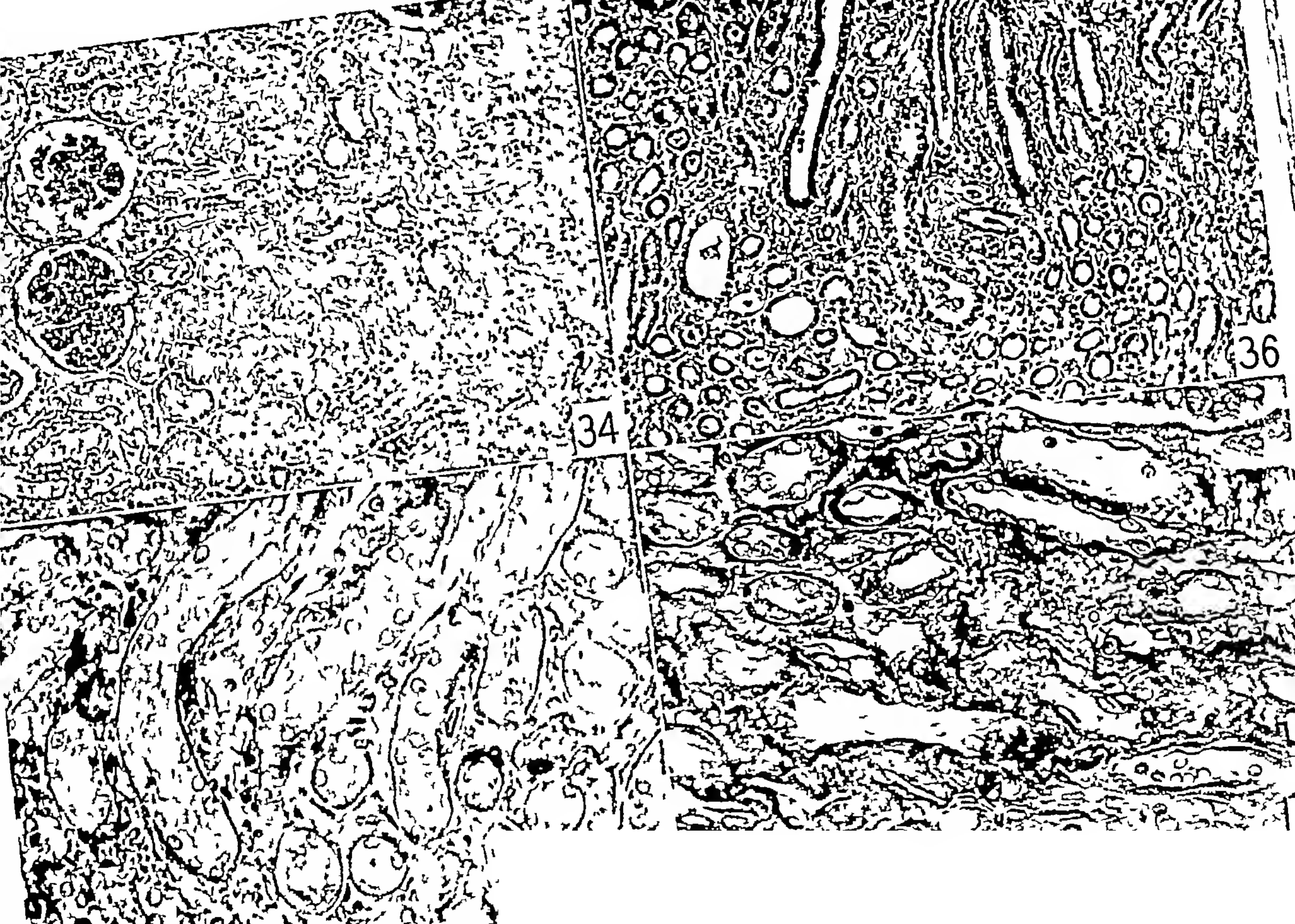
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FIGURE 38

FIG 38 AN EXTENSION OF FIBROUS CONNECTIVE TISSUE FROM THE SUBCORTICAL MEDULLA INTO THE LOWER CORTEX

As seen in Figure 34, the greater part of the cortex was free of any evidence of damage Magnification 150 ×

FIGURE 39

FIG 39 CASE 19, DIED ON THE EIGHTEENTH DAY OF THE DISEASE AND ON THE NINTH DAY OF THE DIURETIC PHASE—AN AREA OF RESOLVED HEMORRHAGE IN THE OUTER MEDULLA SHOWING THE INCREASE IN FIBROBLASTIC CONNECTIVE TISSUE BETWEEN DISTORTED TUBULES—MAGNIFICATION 150 ×

FIGURE 40

FIG 40 CASE 33, DIED ON THE NINETEENTH DAY OF THE DISEASE AND THE TENTH DAY OF THE DIURETIC PHASE—MID-CORTEX

Proximal convolutions are lined with a low and irregular layer of hyperplastic atypical epithelial cells Although the lumen is consequently wide, there is no evidence of thinning of their walls from the tension of dilatation such as occurred in the Oliguric Phase (*cf* Figure 24) Magnification 500 ×

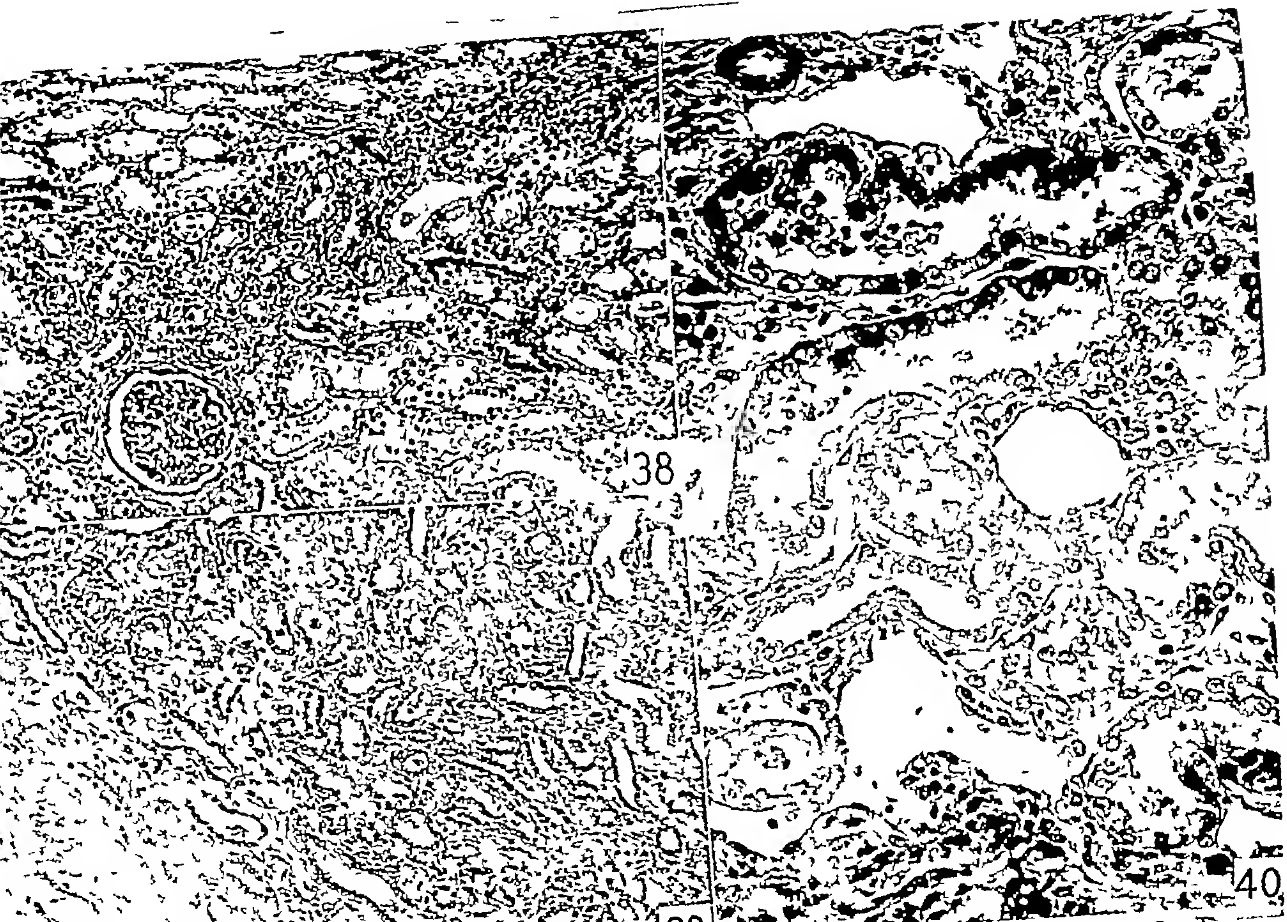

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FIGURE 41

FIG 41 MID MEDULLA SHOWING AN AREA OF HEMORRHAGE SURROUNDING COLLECTING TUBULES WHICH ARE IRREGULARLY DILATED AND DISTORTED IN OUTLINE BY A MARKEDLY HYPERPLASTIC PROLIFERATION OF THEIR EPITHELIUM—MAGNIFICATION 300 ×

FIGURE 42

FIG 42 CASE E, DIED OF CEREBRAL COMPLICATIONS 149 DAYS AFTER THE ONSET OF EHF—THE OUTER MEDULLA SHOWING AN AREA OF MODERATE FIBROSIS IN WHICH COLLECTING TUBULES ARE SEEN WITH A REDUNDANT HYPERPLASTIC EPITHELIUM—MAGNIFICATION 150 ×

FIGURE 43

FIG 43 OUTER CORTEX

Both kidneys showed surface scarring which was more marked in the left. A long wedge-shaped fibrous scar extends from the mid-medulla to the capsule in which there is a heavy round cell infiltration and atrophy of enclosed glomeruli and tubules *Magnification 150 ×*

41

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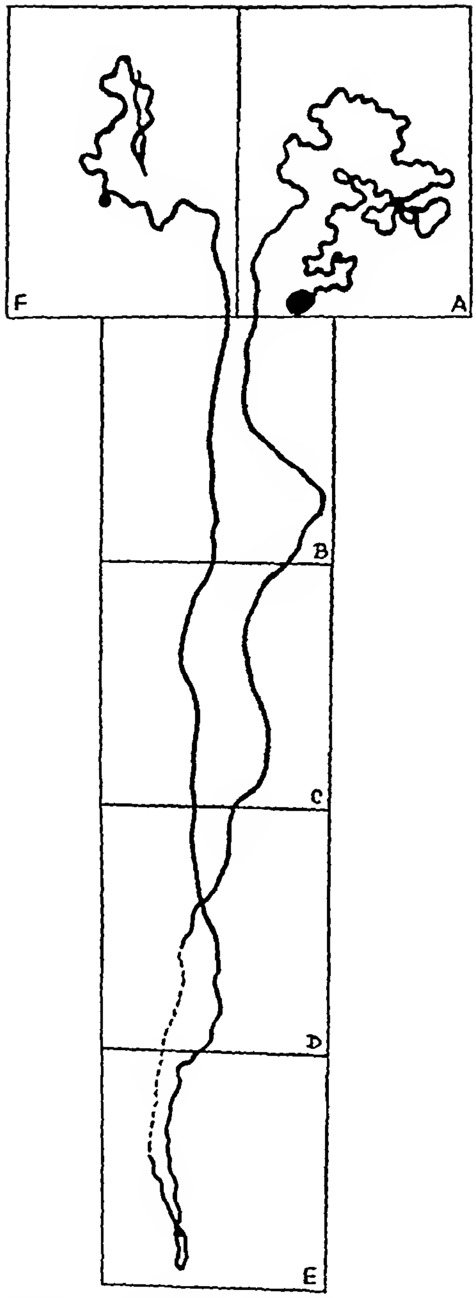


A complete nephron from Case 38 The orientation of the plates is shown in the line tracing The alterations are so slight that this plate may be taken as showing, except where noted, the appearance of a normal dissected nephron stained with iron hematoxylin

1A—The nuclei do not stain and so appear as clear round objects the mitochondrial substance stains heavily, the rodlets and granules individually invisible due to the thickness of the tubule obscure the nuclei in the upper convolutions where, in conformity to the normal gradient, they are most concentrated The glomerulus, dense black due to the mass of its tissue, is normal in configuration the outline of the tuft within the capsule is faintly visible. Original magnification 200 × here reduced to 80 ×

PLATE IB

To the right the terminal segment of the proximal convolution At *a* it enters the subcortical zone of congestion It is slightly compressed and as a result there is some irregularity of staining To the left the ascending limb is moderately dilated and consequently its irregularly thinned wall stains variably


a

PLATE IC

As in IB, the middle third of the terminal segment of the proximal convolution appears more dense than normal due to the pressure of surrounding intertubular congestion

PLATE ID

The termination of the proximal convolution to left, the capillary-like thin portion of Henle's loop was swept away during staining

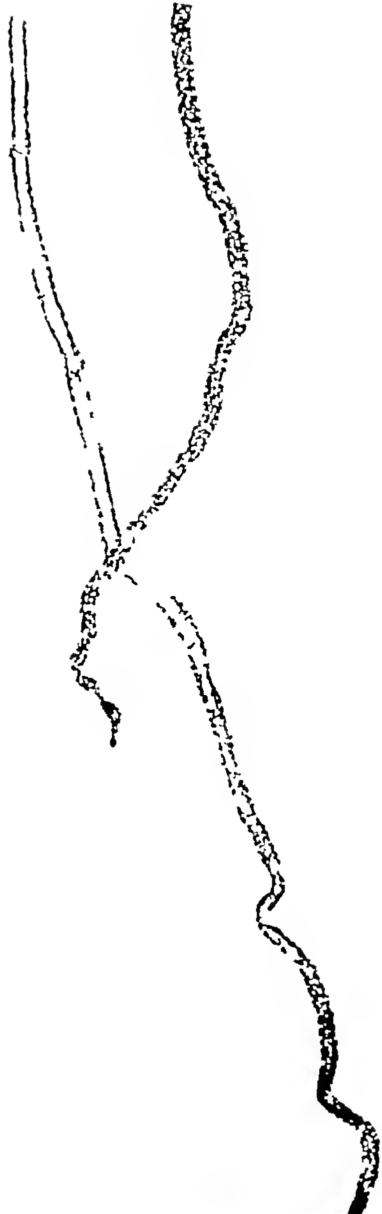


Plate IE

The loop of Henle a remnant of the narrow portion remains and passes into the thick portion which still descending, turns through the loop into the broad ascending limb visible in ID C, and B Two small artifactual breaks are present near the loop

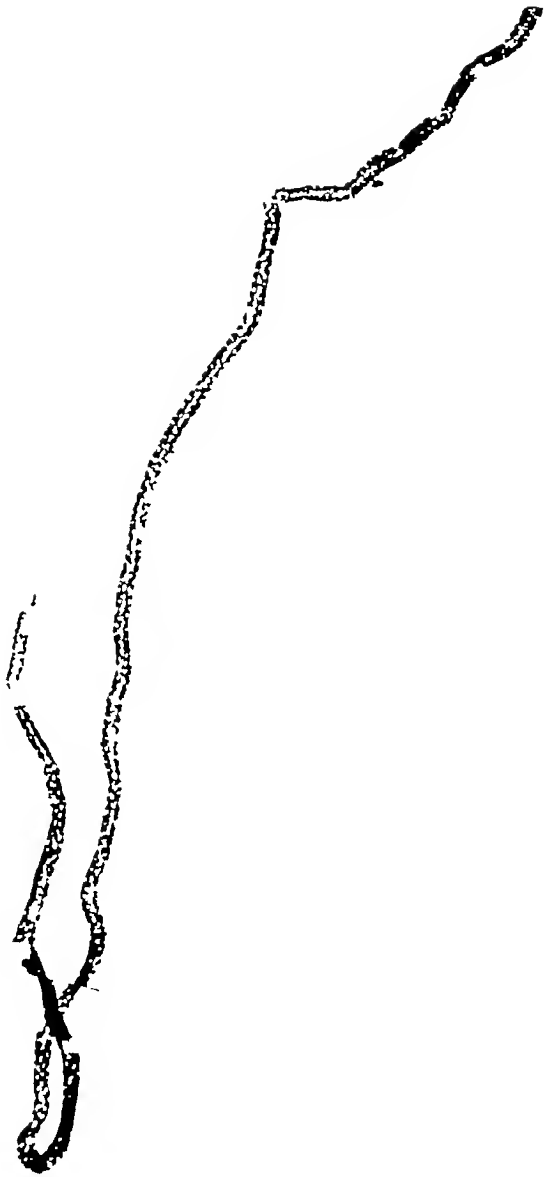
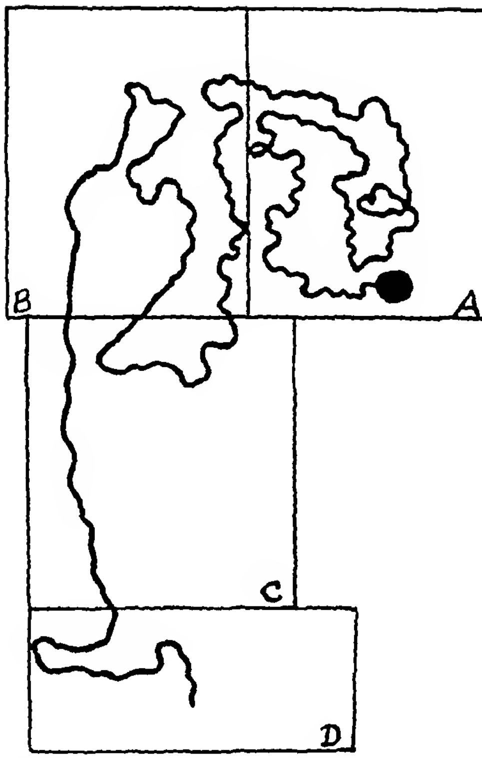


PLATE IF

Passage of the thick portion of the ascending limb into the distal convolution which lay intertwined in the proximal convolutions of IA Except for an irregular dilatation (cf Figure 1) which produces variable staining of its thin wall, the distal convolution is empty and normal in configuration From the top of the plate the connecting tubule, not dilated joins the origin of a peripheral collecting tubule.

Plate III (A to D)



All that remained of a proximal convolution from Case 28 for orientation see outline tracing

IIIA The irregularity of the glomerular contours is due to pressure of the cover glass its configuration was normal Beginning in the first loops of the convolution are irregular stretches of tubule (↑) showing the typical tubulorhexic disruption of anoxia between these the tubule is better preserved (Compare with Figures 20 21 and contrast with Plate IA) Original magnification of 200 ×, is here reduced to 80 ×

PLATE IIIB

To the right, a loop of cortical proximal convolution which entered the zone of subcortical congestion and hemorrhage at *a*, to the left, loops of its medullary continuation show increasing disruptive damage.


a

Plate IIIC. Terminal Medullary Portion of Proximal Convolution Lying in the Zone of Hemorrhage

The entire extent of the tubule is almost continuously necrotic.

PLATE IIID

The terminal medullary segment fades into a wisp of necrotic tubule.

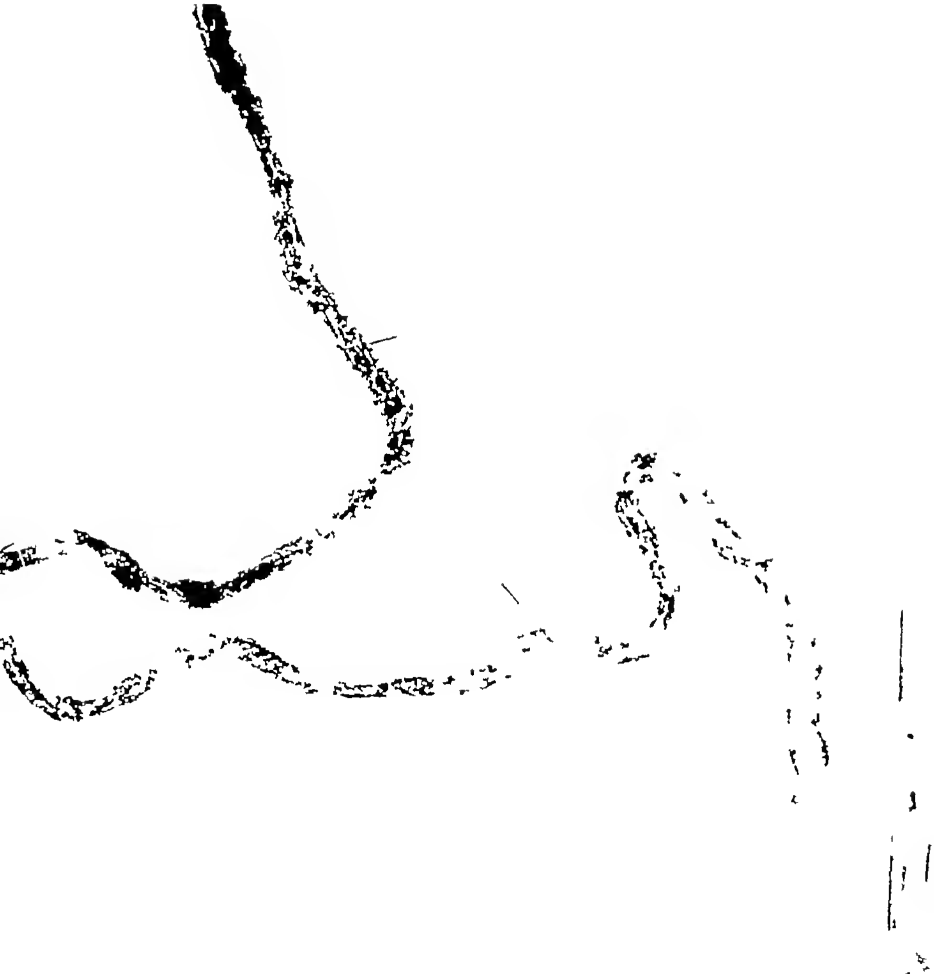


Plate IV A Loop of Henle from the Same Case Lying in the Subcortical Zone of Hemorrhage

Scattered through the length of fairly intact tubule are seen the tubulorhexic lesions of renal ischemia. At *a*, the bend of the loop, the lumen is filled with solid material Original magnification of 100 × is here reduced to 40 ×

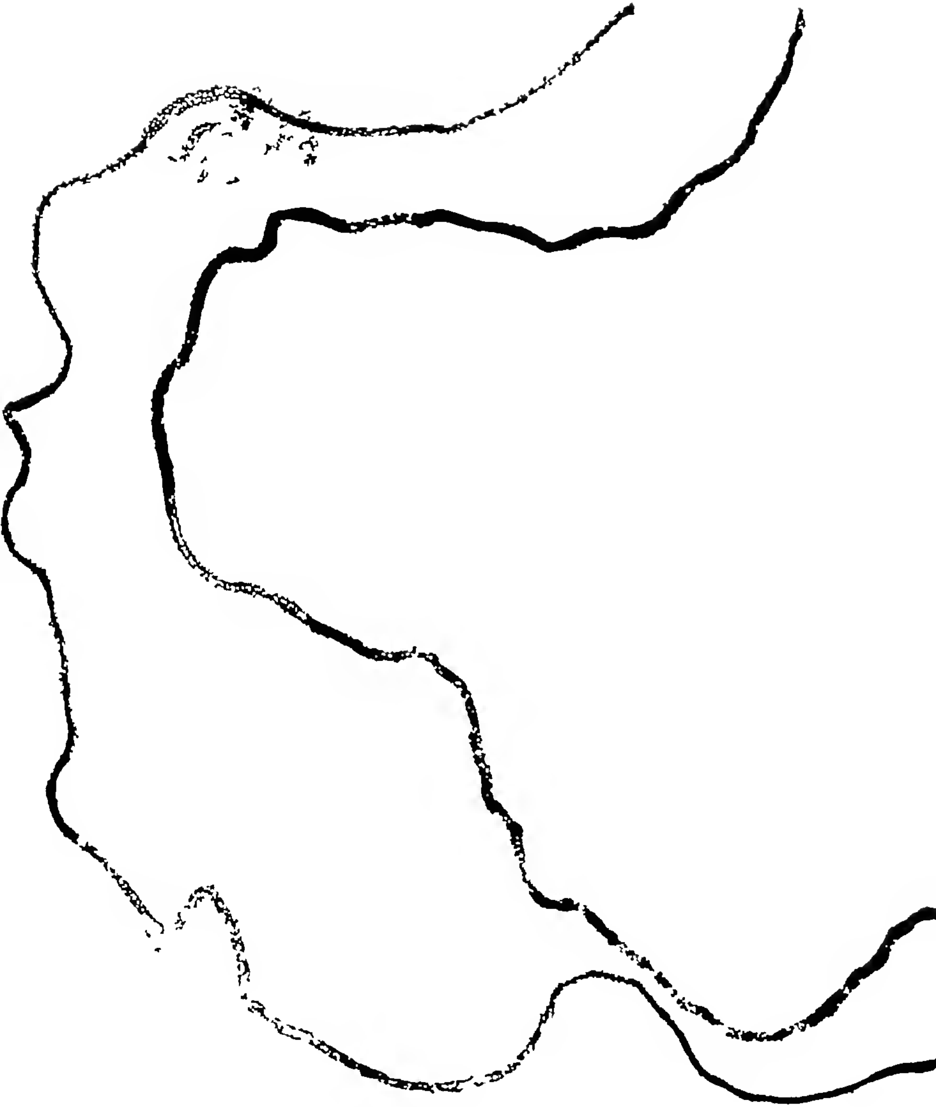
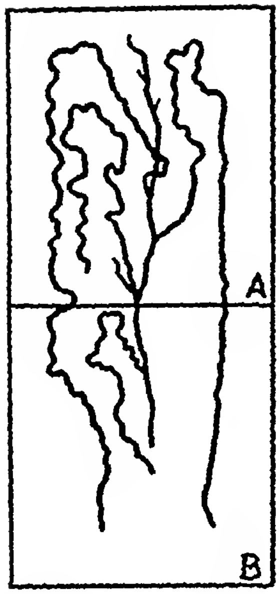


Plate V Collecting Tubules of the Same Case Lying in the Zone of Hemorrhage in the Outer Stripe of the Outer Zone of the Medulla

All show extensive segments of complete tubular necrosis which, with their black stained content of coagulated debris produces a marked irregularity in tubular outline At left, the luminal content is a solid cast, to the right, scattered debris and desquamated cells are visible. Original magnification of 100 × is here reduced to 40 and 20 ×

PLATE VI (A TO B)



Orientation of plates shown in outline tracing From the same case the formation of the peripheral collecting system by the junction of the connecting tubules of 4 nephrons, 4 others have been removed, leaving only their connecting tubules attached.

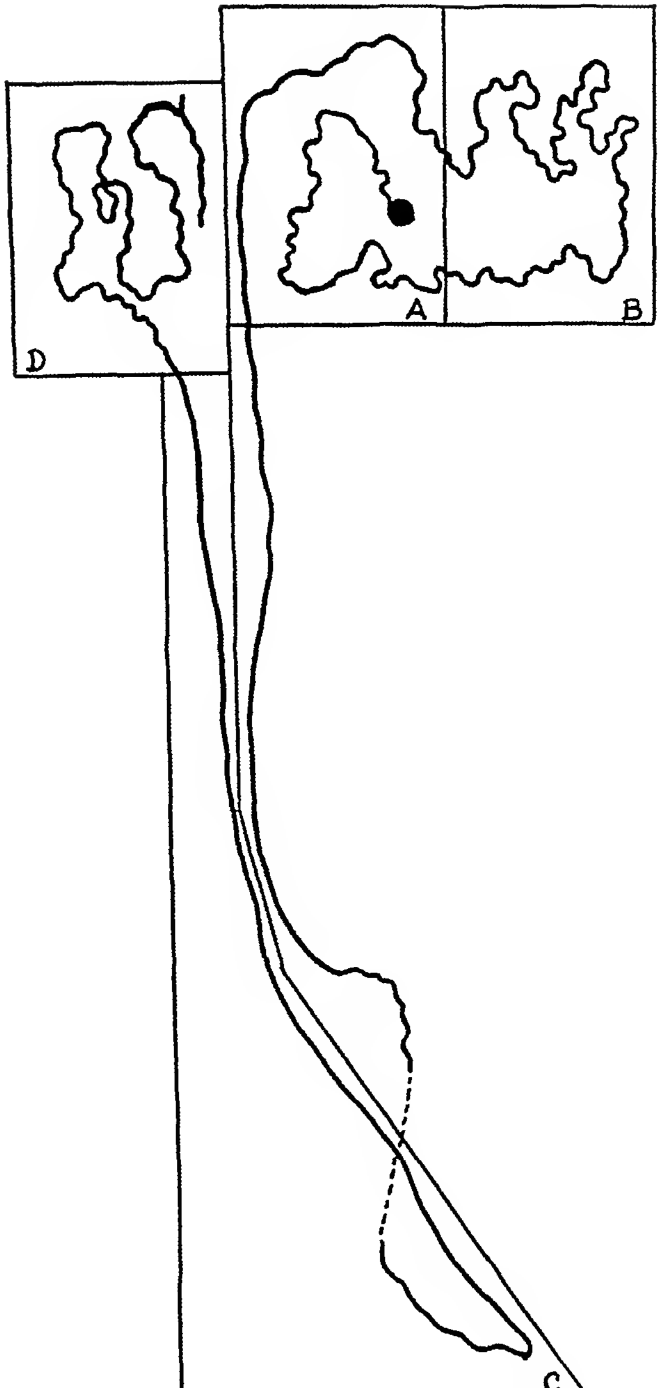
VIA Three distal convolutions (d) filled with coagulum, which stains dense black and so obscures the tubule wall, are moderately dilated The connecting tubules (c) are clear of obstruction, but the central collecting tubule is solid with obstructing material Note that all these tubules, though filled with coagulum, are relatively well preserved as compared to the proximal convolution of Plate III, thus showing the typical distribution of the lesions of ischemia in all forms of Acute Renal Failure. Original magnification of 100 × is here reduced to 40 ×

d'
c'
d
c'
c
d

Plate VIB Continuation of Tubules of VIA

Note the dilatation of the ascending limbs on the left that lead to occluded distals The collecting tubule in the center is filled with deeply stained material

PLATE VII (A TO D)



Orientation of plates shown in outline tracing A nephron from Case 31 in the Phase of Established Oliguria showing dilation throughout its length.

PLATE VIIA THE CORTICAL PORTION OF THE PROXIMAL CONVOLUTION

Bowman's space is distended and the tuft compressed. Note larger afferent arteriole entering the 'polkissen' and a stub of the narrower efferent. After the first coil of proximal convolution the tubule is irregularly distended with resultant patchy thinning of its wall Original magnification of 200 × is here reduced to 80 ×

Plate VIIB Continuation of Dilated Cortical Proximal Convolution
Note the irregular thinning of the epithelial pattern in the distended portions

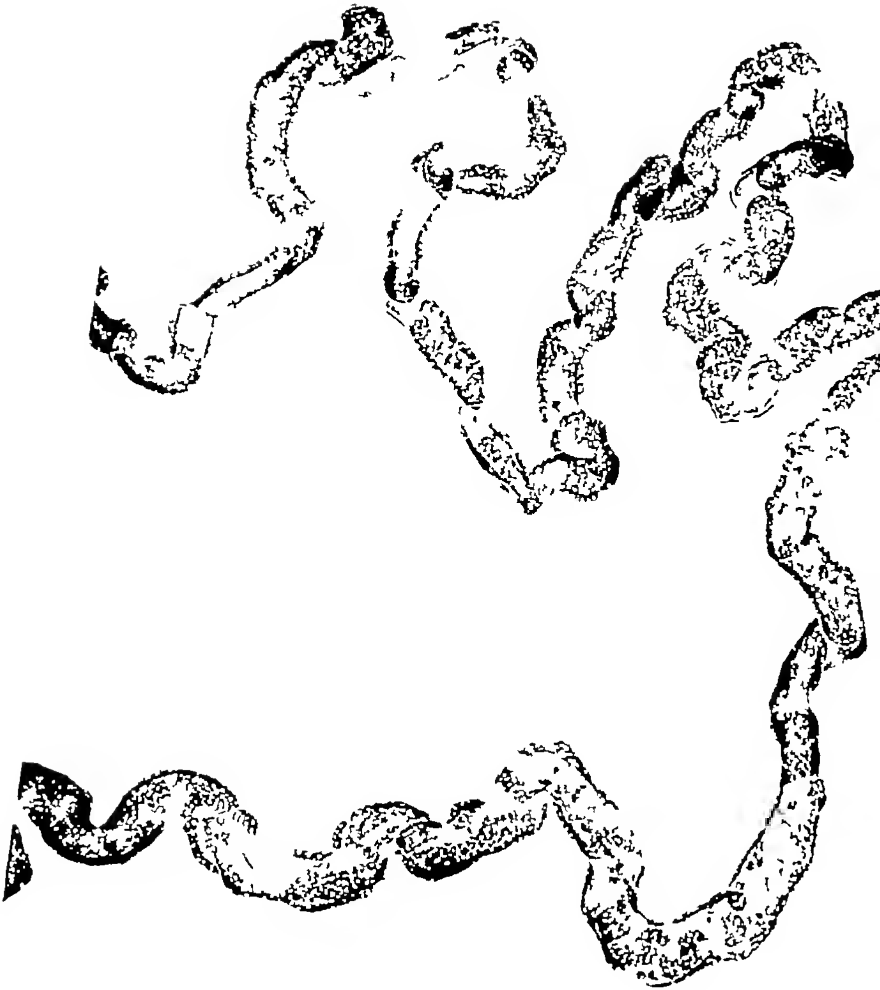
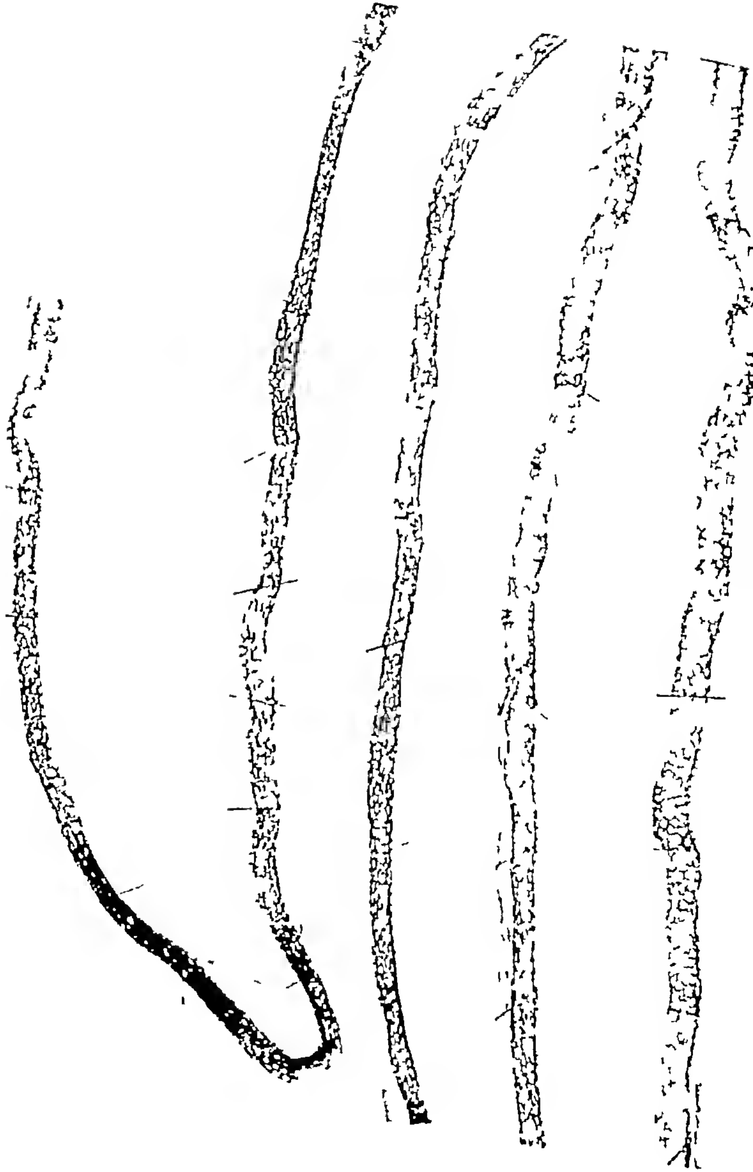


Plate VIIC Portions of Dilated Ascending Limb and Loop

The tubule is well preserved in spite of distention



ATE VIID The Dilated Distal Convolution, Containing Some Dark Staining Debris, the Connecting Tubule and Collecting Tubule All Well Preserved

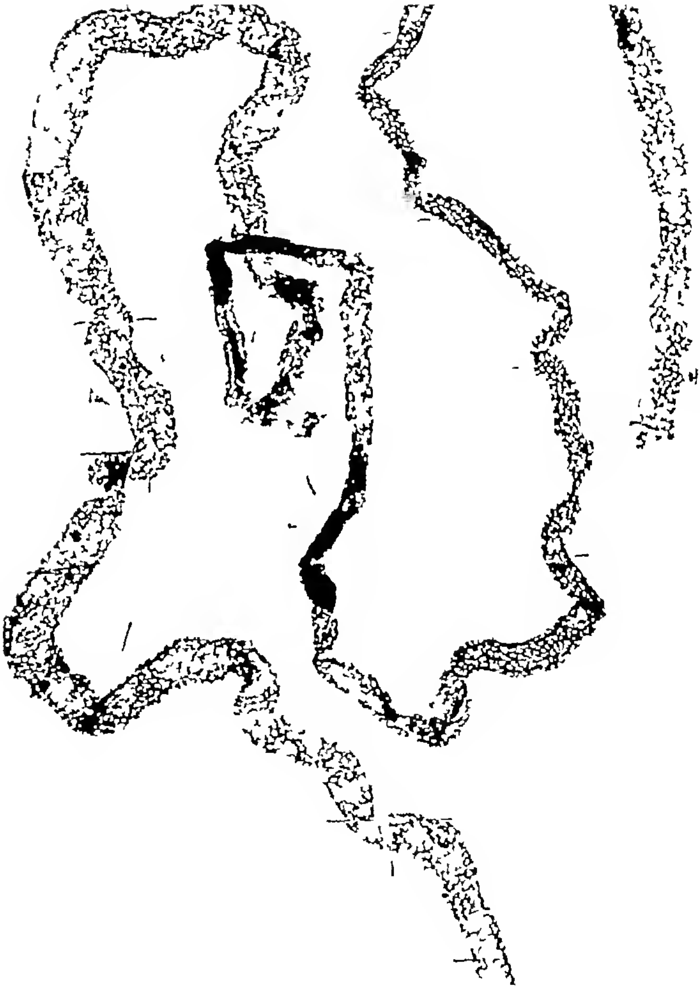
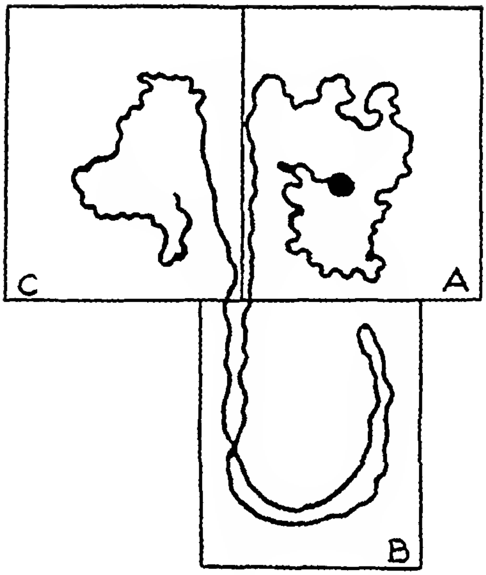


Plate VIII (A to C)



The tracing shows the orientation of the three plates of a complete cortical short looped nephron from Case D, who died in the period of transition from oliguria to diuresis

Plate VIIIA The glomerulus is of normal configuration, the dark spot on the afferent arteriole is a collection of "myo epithelial" cells of the juxta-glomerular apparatus The greater part of the proximal convolution is dilated. As a result of atypical regeneration the epithelium of its wall is irregular in thickness, and redundant. For histological appearance *cf* Figures 32 and 33 At *a* there is an incompletely healed tubulorhexic lesion There are many desquamated epithelial cells in the lumen of the convolution shown to the left. Original magnification of 200 × is here reduced to 80 ×


a

Plate VIIIB

To the right the continuation of the proximal convolution which, dilated and containing desquamated epithelial cells ends at *a* At *b* the thin portion of the loop passes to the thicker portion and through the loop ascends toward the distal convolution

a

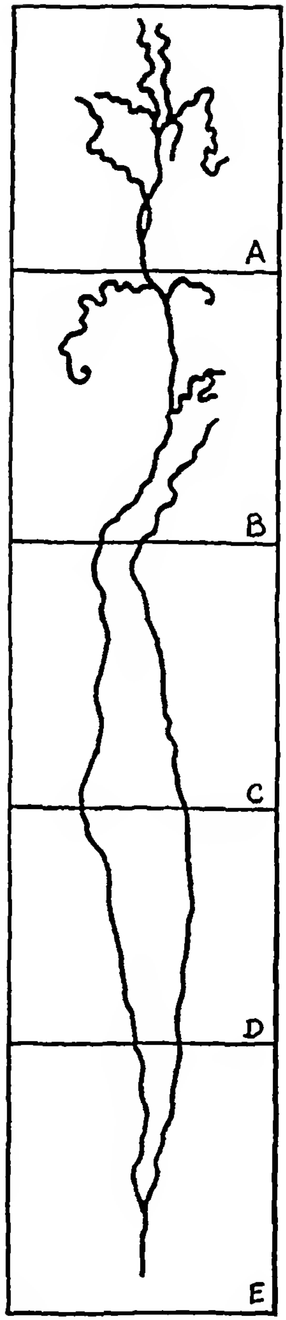
Plate VIIIB

To the right the continuation of the proximal convolution which, dilated and containing desquamated epithelial cells ends at *a* At *b* the thin portion of the loop passes to the thicker portion and through the loop ascends toward the distal convolution


b
a

Plate VIIIB

To the right the continuation of the proximal convolution which, dilated and containing desquamated epithelial cells ends at *a* At *b* the thin portion of the loop passes to the thicker portion and through the loop ascends toward the distal convolution.



From the same case. Orientation of plates in line tracing

PLATE IXA ORIGIN OF THE PERIPHERAL COLLECTING TUBULE SYSTEM

Five connecting tubules all filled with deeply stained coagulated material, but intact, which lay beneath the capsule in the outer cortex. The cellular pattern of the collecting tubule is normal and it is not filled with coagulum. Original magnification of 200 × is here reduced to 80 ×

PLATE IXB THE SAME COLLECTING TUBULE IN MID-CORTEX

Three more connecting tubules join the main tubule which, as shown by its clear cellular pattern is intact and empty To the lower right, a neighboring collecting tubule

Plate IXC Continuation of the Two Collecting Tubules into the Subcortical Area of Hemorrhage in the Outer Zone of the Medulla

Below the level *a*, the damage to the epithelial cells is barely apparent in the loss of clarity of the nuclear pattern in the tubule to the left (*cf* see Plate IXB), and is obvious in that to the right.

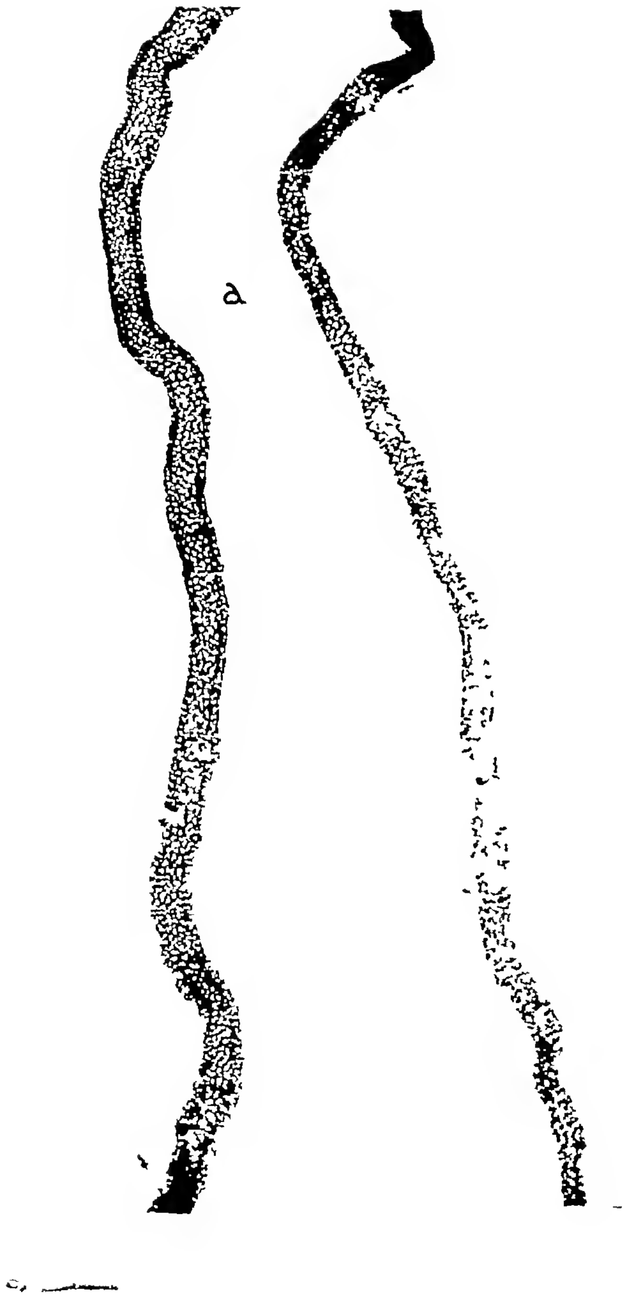

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Plate IXD Continuation of the Two Collecting Tubules with Extensive Epithelial Necrosis of the Greater Part of Both

PLATE IXE CONTINUATION OF THE TWO COLLECTING TUBULES INCLUDING THEIR JUNCTION IN MID-MEDULLA

Only the external configuration of the two tubules, now entirely necrotic, remains After the tuning-fork junction the tubule continues, necrotic and filled with deeply stained debris For histological appearance of similar necrotic collecting tubules, *cf* Figure 29

PLATE X (A to B) Portion of Proximal Convolution from Case 33 Who Died on the Nineteenth Day of His Illness and in the Tenth Day of Diuresis

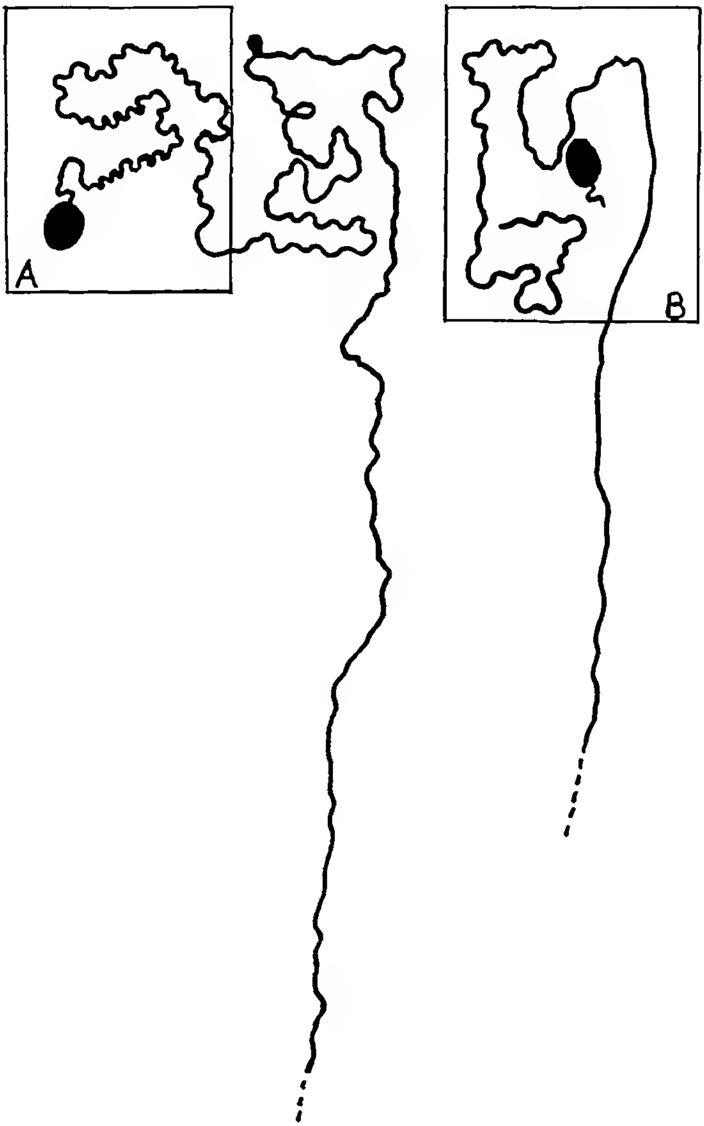

Plate XA

Although the lumens of the proximal convolutions appear large in histological section (*cf* Figure 40) as can be seen from the dissected specimen this is not due to dilatation of the tubule but to the irregular regeneration of its epithelial wall (*a*) Original magnification of 200 × is here reduced to 80 ×

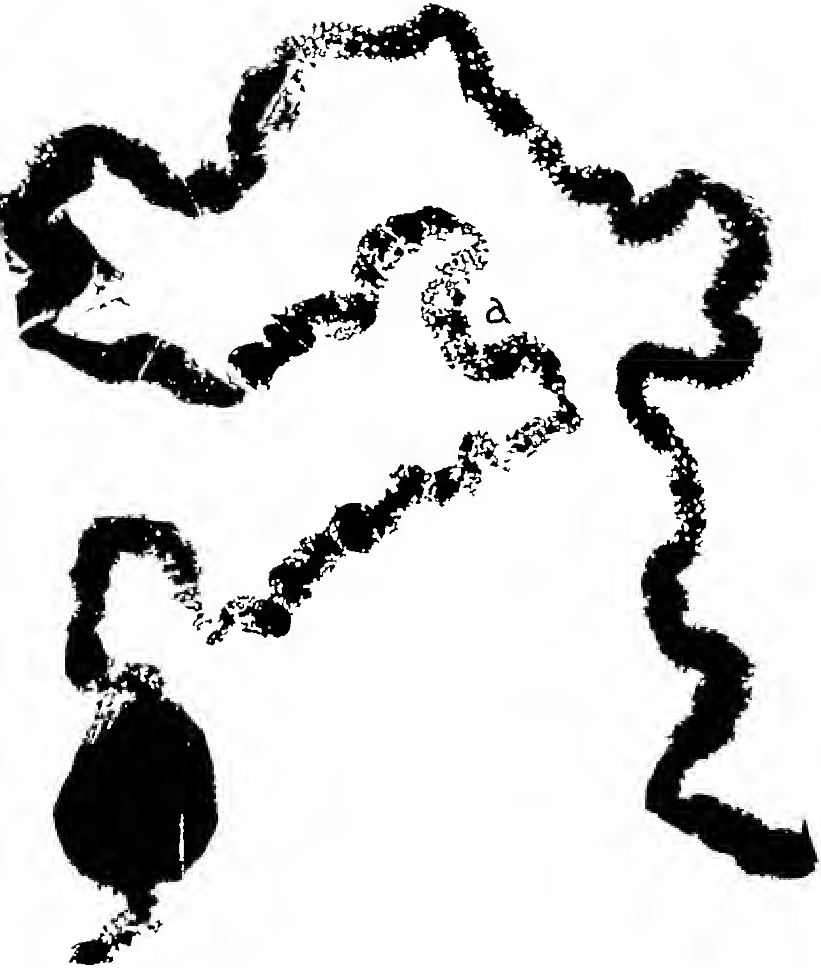
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Plate XB The Distal Convolution of the Same Nephron

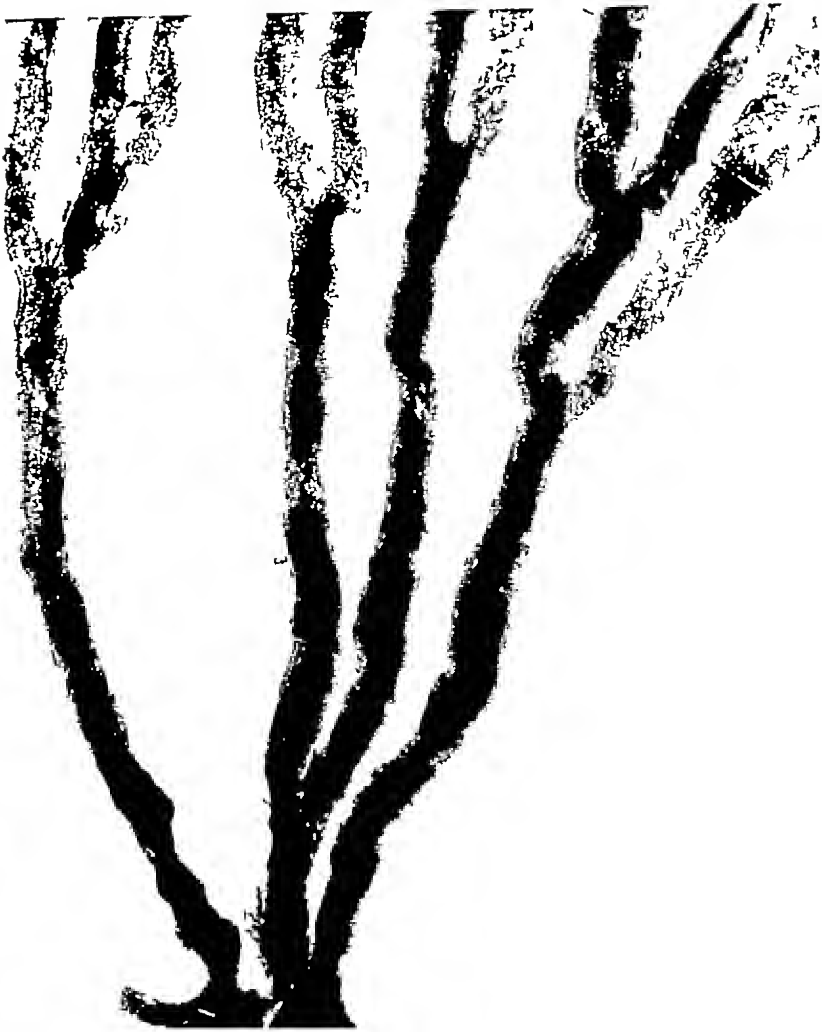
A duplicate print of the glomerulus has been mounted in the position of its attachment to the tubule The ascending limb is essentially normal The first half of the distal convolution is irregularly dilated the second half filled with a large solid cast-like mass which continues into the connecting tubule.

Plate XI (A to B) The Terminal Collecting Tubule and the Ducts of Bellini from the Same Case

In the normal kidney these tubules show smooth, even contours with gradually increasing diameters These are markedly irregular both from the presence of the large renal failure casts of Addis that intermittently distend their lumen and from the irregular hyperplastic proliferation of their epithelial cells Original magnification of 100 × is here reduced to 40 ×

PLATE XIB THE DUCTS OF BELLINI ENTERING THE RENAL PELVIS

The foot like appendage that joins the three ducts below is a reflection of the pelvic epithelium which usually remains attached in spite of dissection As has been observed, the tortuosity and rregularity of contour is seen to be due to the masses of debris that fill the lumens as well as o the marked hyperplasia of epithelial cells of the walls of ducts For histological appearance f Figure 41 Original magnification of 175 × is here reduced to 80 ×



ELECTROPHORETIC STUDIES OF RED CELL EXTRACTS OF STORED BLOOD[1]

By EDWARD R. BERRY AND ALFRED CHANUTIN WITH THE ASSISTANCE OF CLAUDE A. SMITH

(*From the Department of Biochemistry School of Medicine University of Virginia Charlottesville Va*)

(Submitted for publication March 5 1956 accepted October 10 1956)

It has been shown that red cells of blood collected in acid-citrate dextrose (ACD) undergo changes in their dimensions osmotic fragility permeability and metabolism during storage at 4° C (1) Information concerning the effect of storage on the physical and chemical states of soluble red cell proteins particularly hemoglobin, is not available Such data may be of interest in view of the marked changes observed in the mobility and concentration of several proteins during the storage of plasma (2) This paper presents evidence from electrophoretic analyses that the concentration of components of extracts of red cells is changed during storage of blood

METHODS

Healthy male medical students about 21 years of age, and anemic hospitalized patients served as donors Sterile precautions were taken in the collection storage and sampling of the blood As a rule 50 ml. of blood was drawn from each individual before breakfast and collected in a 125 ml. cotton plugged, sterile, Pyrex Erlenmeyer flask containing 12.5 ml. ACD (NIH Sol. B) Sterilized ACD-inosine or ACD adenosine solutions were also used in the initial collection of blood. Stored blood was supplemented by calculated amounts of nucleoside in a sterilized saline solution. Phenergan® (phenothiazine, 10 (2 dimethylaminopropyl) hydrochloride) dissolved in saline, was sterilized by filtration through an ultra fine fritted Pyrex glass disc, and the required amounts were added to the sterile ACD solution After removing a 5-ml. aliquot of blood for analysis the remaining blood was stored at 4 C. It was necessary to withdraw 10 ml aliquots for analysis if there was an excessive loss of red cells during the washing procedures or whenever the hematocrit values of the blood were low

The blood sample was centrifuged, the plasma and the top cellular layer were removed the cells were washed and lysed at room temperature and clarified by centrifugation in the refrigerator according to Drabkin's procedure (3) The first step involved washing once with 0 9 per cent NaCl and three times with a 1.2 per cent NaCl-0 0025 M $AlCl_3$ mixture. Clarified solutions were examined under oil immersion with a phase microscope without observing stroma or stroma filaments. The hemoglobin concentration of the clarified red cell extract was determined (4) and an appropriate volume was diluted with distilled water to yield 5 ml of a 1.2 or 1.4 per cent hemoglobin solution. This solution was dialyzed, in the cold, against 0 05 M sodium cacodylate cacodylic acid buffer (pH 6.5) which was changed three times during a 24 hour period. Electrophoresis was done in a Klett Model of the Tiselius apparatus using a microcell of 2 ml capacity The temperature was maintained at 2.0 ± 0 01 C. Electrophoresis was allowed to proceed for 88 minutes with an open anode vessel and the boundaries were compensated (without interrupting the current) to the center of the cell the run was continued for 30 additional minutes with a closed anode vessel Photographs were taken by Longsworth's scanning technique (5) using CTC panchromatic plates and a Wratten No 25 filter These patterns were better defined than those obtained with the Philpot Svensson cylindrical lens method. The ascending patterns were analyzed by dropping lines at the minima between boundaries (6) and where there was no well-defined minimum the same relative position of the partitioning line was maintained. The patterns of the descending limb were not analyzed because the boundaries were poorly separated.

The tests for osmotic fragility were similar to those recommended by Schales (7)

Adenosine and inosine were purchased from the Nutritional Biochemicals Corporation and from the Schwarz Laboratories Inc. and the Phenergan® was obtained from Wyeth Inc.

RESULTS

Conditions for electrophoresis of red cell extracts

Aliquots of a red cell extract were analyzed by electrophoresis in cacodylate buffer solutions (pH 6 5) with molar concentrations of 0 1 0 075, 0 05 and 0 025 Under these experimental conditions a single boundary is obtained with the 0 1 M buffer The pattern obtained with 0 075 M buffer shows a well-defined small and a large component

[1] This investigation was supported by the Medical Research Development Board, Office of the Surgeon General Department of the Army under Contract No. DA-49-007 MD 160

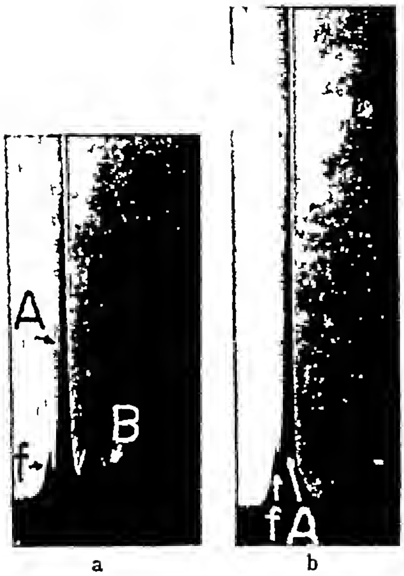


FIG. 1. ELECTROPHORETIC PATTERNS OF RED CELL EXTRACTS

(a) Freshly drawn blood.
(b) ACD blood stored 50 days.

and a small shoulder on the leading edge of the front component. Three well-defined boundaries (f, A and B) are observed in the 0.05 M buffer (Figure 1, a). The pattern of the 0.025 M buffer aliquot was difficult to analyze because of excessive sharpening of the boundaries. Aliquots of a red cell extract were adjusted to 1.25 per cent total hemoglobin and were analyzed at pH 6.4, 6.5 and 6.6 without showing significant differences in the distribution of the components. In addition, solutions varying in concentration from 0.75 to 1.75 per cent hemoglobin were analyzed at pH 6.5 and the results were similar to those observed with the 1.25 per cent solution. As a result of these preliminary experiments, the red cell extracts were routinely analyzed in a 0.05 M cacodylate buffer at pH 6.5 between 1.25 and 1.5 gm. per cent hemoglobin. Under these conditions, a steady electrophoretic state, as defined by Hoch (8) and by Nichol (9), is obtained.

ACD controls

The patterns of red cell extracts of freshly drawn ACD bloods from male students were analyzed by the Philpot-Svensson method. The concentration for component B was determined as per cent of the total area for the three components. The average per cent concentration of component B for 12 subjects was 24.5 with minimum and maximum values of 22.0 and 26.7, respectively. The Longsworth scanning procedure gives values which are 6 to 10 per cent greater than by the Philpot-Svensson method.

Effect of storage

ACD and ACD plus Phenergan®, inosine and adenosine. The effects of Phenergan®, inosine and adenosine on the concentration of component B and on the degree of hemolysis in 0.6 and 0.85 per cent NaCl were determined on a single sample of blood in order to evaluate their relative effects (Figures 2, 4, 6, 7). A unit of blood (480 ml.), obtained from a healthy young man (E. S.), was collected in 120 ml. ACD (NIH Sol. B) and aliquots were supplemented with the test materials.

The values for the percentage distribution of boundaries f, A and B are subject to errors inherent in the Tiselius-Kabat line-dropping procedure (6) and in the dissymmetry of the boundaries. Inspection of pattern b in Figure 1 indicates that component B is not present. Nevertheless, the line-dropping procedure makes it necessary to designate about 10 per cent of the total pattern as component B, when in reality most, if not all, of this area represents the trailing foot of component A. In presenting results for changes in terms of per cent of the original component B concentration, values of 30 to 40 per cent may be

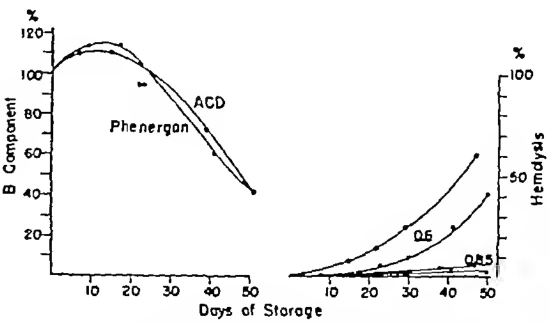


FIG. 2. EFFECT OF STORAGE OF ACD BLOOD SUPPLEMENTED WITH PHENERGAN® (0.4 MM PER L. BLOOD) ON COMPONENT B CONCENTRATION AND ON OSMOTIC FRAGILITY

The total hemoglobin concentration of the red cell extracts in this and subsequent experiments was about 1.2 per cent. The figures 0.6 and 0.85 in this and other figures refer to the percentage concentration of NaCl.

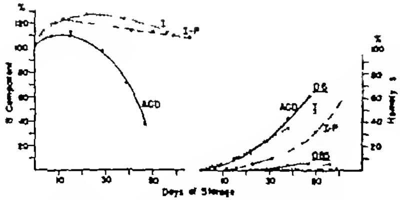


FIG 3 EFFECT OF STORAGE OF ACD BLOOD SUPPLEMENTED WITH INOSINE (I) AND INOSINE AND PHENERGAN® (I–P)

Inosine (2,500 μM per 100 ml RBC) and Phenergan® (0.4 mM per L. blood) were added immediately after collection of blood (E. S)

encountered for patterns having practically none of this material present

Phenergan® The observation of Schales (7) that Phenergan® retarded the rate of increase of osmotic fragility of red cells in 0 6 per cent NaCl of ACD stored blood is confirmed as shown in Figure 2 The changes in the concentrations of component B in stored ACD and ACD-Phenergan® blood are about the same There appears to be no relationship between the osmotic fragility and the distribution of the components of the red cell extracts in these experiments

A study of the effect of another phenothiazine derivative, chloropromazine, was discontinued because of its hemolytic properties

Inosine Donohue Finch and Gabrio (10) implicated inosine the product of adenosine deamination, as the agent which is effective in erythrocyte preservation and Gabrio Donohue, Huennekens and Finch (11) demonstrated that this nucleoside prolonged the viability of the red cell Data plotted in Figure 3 show the changes in the concentration of the component B in (a) ACD control (b) inosine and (c) inosine Phenergan® supplemented aliquots of a single blood during storage The concentration of component B remains elevated for more than 60 days in the inosine-containing aliquots In contrast, the values for this component in the ACD aliquot increase during the first few weeks and subsequently decrease at a comparatively rapid rate The osmotic fragility of the inosine Phenergan® red cells in 0 6 per cent NaCl is much lower than that of the inosine and ACD samples The values for the per cent hemolysis after 47 days of storage of aliquots a b and c are 61, 43 and 28 respectively

Inasmuch as the addition of inosine plus Phenergan® to ACD blood had a profound effect on maintaining a high component B concentration and low osmotic fragility during storage additional data were obtained with samples of blood from three different donors (Figure 4) Considerable individual variations are noted in these experiments The component B concentrations remained elevated during periods of about 40 to 60 days It will be noted that the extent of hemolysis in 0 6 per cent NaCl is related to the time that the concentration of component B remains elevated

Experiments were designed to determine the influence of varying concentrations of inosine on the electrophoretic patterns of stored blood In Figure 5 results are shown for three individual ACD bloods supplemented with 1) 2 500 μ moles 2) 1,250 μ moles and 3) 625 μ moles inosine per 100 ml of red cells The data for bloods 2 and 3 indicate that the rate of decrease in component B concentration is greater than that of ACD blood (Figure 2) After storage for 48 days 2,500 μ moles and 1,250 μ moles of inosine per 100 ml. of red cells were added to bloods 3 and 2 respectively The results are striking since the concentration of component B of blood 3 is elevated to the control level and remains high during the period of observation The value for blood 2 is also elevated from about 40 to 85 per cent but

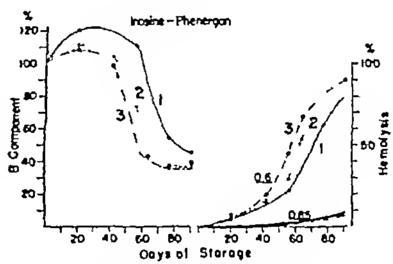


FIG. 4 EFFECT OF STORAGE OF ACD SUPPLEMENTED WITH INOSINE PLUS PHENERGAN®

Curves 1 2 and 3 represent data for bloods from three healthy male donors. Concentrations of inosine and Phenergan® are the same as those in the previous experiment (Figure 3)

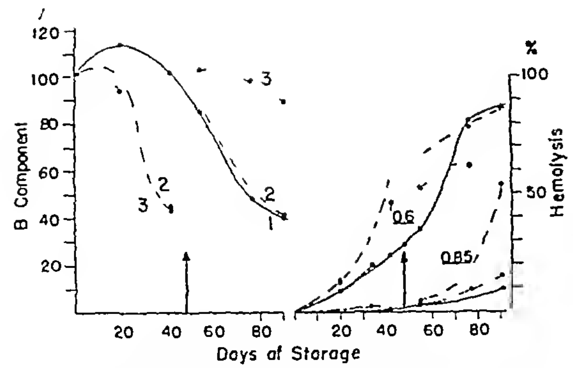


FIG 5 EFFECT OF STORAGE OF ACD BLOOD SUPPLEMENTED WITH VARYING AMOUNTS OF INOSINE

Inosine was added to three different bloods when drawn Sample 1 2 500 μ moles sample 2, 1,250 μ moles, and sample 3, 625 μ moles per 100 ml RBC After samples 2 and 3 were stored for 48 days 1,250 and 2 500 μ moles inosine per 100 ml RBC were added to the respective bloods

subsequently decreases at the same rate observed for blood 1

The values for the per cent hemolysis in 0 6 per cent NaCl of red cells of blood 1 are markedly increased after about 50 days of storage and practically all the cells are hemolyzed after 90 days The osmotic fragilities of the red cells of bloods 2 and 3 are similar but are appreciably greater those of blood 1 during the first 40 days of ...e The additional supplement of 2,500 μ ...s of inosine to blood 3 prevents the marked increase in osmotic fragility On the other hand, addition of a smaller amount of inosine to blood 2 has a temporary effect The increased values for the per cent hemolysis of red cells of blood 2 in isotonic saline after the second addition of inosine probably reflect the lack of optimal amounts of nucleoside, which is undoubtedly concerned with the integrity of the stroma

Adenosine Component B concentration of ACD blood supplemented with adenosine remains elevated during a 60-day period of observation (Figure 6) It will also be noted that these red cells are more readily hemolyzed than those of the control ACD blood The addition of adenosine or inosine to ACD blood stored for 22 days is responsible for maintaining a prolonged, elevated component B concentration and for a decreased osmotic fragility

Effect of temperature Three aliquots of the ACD blood were stored at 4°, 20° and 37° C The results in Figure 7 show that the storage temperature has a pronounced effect on the concentration of component B and on the osmotic fragility After 10 hours of storage at 37° C about 60 per cent of the original concentration of component B disappears A similar but not as marked a change is observed after storage at 20° C Osmotic fragility is increased within 48 hours after storage at 37° C, a corresponding value is observed at the end of 8 days of storage at 20°

Bloods from anemic patients The data in Figure 8 show the changes in component B concentrations during storage of 13 bloods from patients with anemia of primary and secondary origin The rapid decrease in values for red cell extracts from patients 1 to 5 is unusual and striking The data for patients 6 to 8 fall within the range observed for control ACD blood during the first 30 days of storage The values for cases 9 and 10 remain at a constant level during the period of observation An unusually high concentration (36 per cent) of component B values for the erythrocytes of patients 11, 12 and 13 is

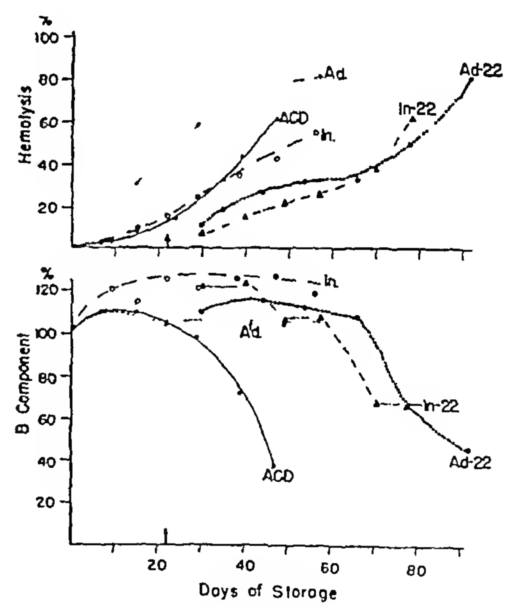


FIG 6 EFFECT OF STORAGE OF ACD BLOOD SUPPLEMENTED WITH ADENOSINE (Ad) AND WITH INOSINE (In)

At 0 days, a suspension of adenosine (2,500 μ moles per 100 ml RBC) was added to the blood (E S) After storage of ACD blood for 22 days, adenosine (2,500 μ moles) was added and incubated for 1 hour at 37° C Inosine was added to blood under the same conditions

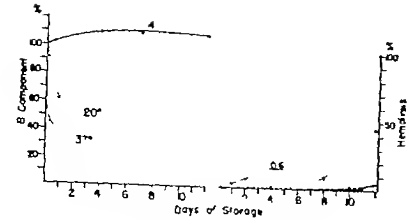


FIG 7 EFFECT OF STORAGE OF ACD BLOOD (L S) AT 4 20 AND 37 C

striking This is particularly true for the case of lymphocytic leukemia in which the initial concentration of component B (17 7 per cent) was the lowest value observed in these experiments

The osmotic fragilities of the red cell in 0.6 per cent NaCl from these anemic patients tend to be consistently below the values observed for red cells from healthy individuals The lack of any relationship between the behavior of component B and osmotic fragility is apparent in this group

DISCUSSION

Evidence for the heterogeneity of hemoglobin has been presented by a number of investigators Geiger (12) first demonstrated the presence of two different hemoglobins in the red cell of a number of species He found that the hemoglobins could be separated most effectively if a red cell extract was subjected to cataphoresis at a low ionic strength Additional evidence was provided by solubility studies (13) by electrophoresis (14–16) by oxygen capacity measurements (17 18) by chromatographic separation (19–21) and by stability to alkaline denaturation (22–27) Schapira Dreyfus and Kruh (28) were able to prove the existence of at least two hemoglobins after treating extracts of Fe^{59} labelled red cells of a single blood by alkali denaturation, electrophoresis paper chromatography and alumina chromatography In addition, globin prepared from hemoglobins of several species was found to consist of two components (29 30) Data presented in this paper show the presence of at least two pigmented boundaries or components in red cell extracts which are assumed to be hemoglobins

The identity of each of the three boundaries observed in the electrophoretic patterns of red cell extracts is not known The fast moving boundary (component f) can be observed as a colorless material and probably represents the concentration gradient of the cacodylic acid mixture Experiments to test this were conducted by comparing the areas of component f after electrophoresing aliquots of a red cell hemolysate in 0 05 M buffer into 0 047 0 050 and 0 053 molar cacodylate buffers (pH 6 5) in the ascending limb The areas of this boundary were smaller in the 0 047 and greater in the 0 053 molar solutions which indicates that this boundary is due to the cacodylate ion Components A and B are deeply pigmented boundaries which are assumed to be hemoglobins

Derrien and Reynaud (14) observed heterogeneity in 0 1 M cacodylate (pH 6 5) if electrophoresis was continued for a sufficiently long period In the present experiments a single boundary was obtained for red cell extracts when electrophoresis was done at pH 6 5 in 0 1 M cacodylate buffer for two hours and heterogeneity was encountered in 0 075 M and lower concentrations Slater and Breyer (16) believe that hemoglobin ... in several different elementary units ... strengths During the storage of

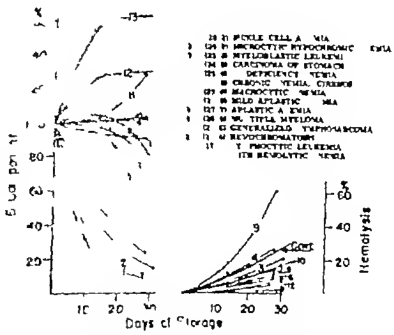


FIG. 8 EFFECT OF STORAGE OF BLOOD (ACD) OF PATIENTS WITH ANEMIA

The patterns for these bloods were obtained with the Philpot Svensson cylindrical lens method. The numbers in parentheses represent the 0 day concentration of component B these values are designated as 100 per cent. The stippled area represents the range of values for component B concentration of 12 bloods from healthy young men.

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ANNOUNCEMENT OF MEETING

The 49th Annual Meeting of the American Society for Clinical Investigation will be held in Atlantic City New Jersey, on Monday, May 6, 1957, with headquarters at the Chalfonte-Haddon Hall The scientific session will begin at 9 A M at the Steel Pier Theater

THE BIOSYNTHESIS OF THE FATTY ACIDS OF THE PLASMA OF MAN I THE FORMATION OF CERTAIN CHROMATOGRAPHICALLY SEPARATED HIGHER FATTY ACIDS OF THE MAJOR LIPIDE COMPLEXES FROM ACETATE-1-C^{14} [1]

By S. R. LIPSKY A. HAAVIK, C L. HOPPER, and R. W McDIVITT with the technical assistance of BARBARA M. MOSSBERG

(*From the Department of Medicine Yale University New Haven Conn*)

(Submitted for publication July 2, 1956 accepted October 17 1956)

One of the outstanding difficulties precluding ie use of radioactive tracers for a detailed study f the dynamics of lipide metabolism in man has een the lack of adequate micromethods for the olation and identification of the major lipide omplexes present in the plasma and the individual itty acids associated with these groups. In re 'nt years with the advent of new chromato- raphic procedures two significant achievements ave occurred in this sphere By the use of silicic cid columns Borgström (1, 2) in 1952 and illerup and Mead (3) in 1953 separated lipide xtracts into sterol ester triglyceride, free fatty cid and phospholipide fractions Crombie, ,omber and Boatman (4) extended the reverse hase partition chromatographic technique of Howard and Martin (5) and while encountering onsiderable overlapping of zones demonstrated e separation of milligram quantities of certain mmon saturated and unsaturated fatty acids ntained in natural mixtures

The present investigation was undertaken in an ort to combine and modify these methods in er to determine (a) the nature and quantity he specific higher saturated and unsaturated y acids that form ester linkages with the lipide plexes of the plasma of man and (b) the rates nthesis of these acids following the adminis- on of acetate-1 C^{14}

METHODS AND MATERIALS

. *The separation of the total lipide extract of the na into the total fatty acids of the sterol esters riglycerides and the phospholipides*

Extraction. Four patients with limited life expect lue to carcinoma of the lung without evidence of metastases were selected for study All were considered to be in a good nutritional state and clinically free from gross metabolic disturbances. The subjects were main tained on normal hospital diets during the course of the investigation. Two hundred microcuries of acetate 1 C^{14} (specific activity 1 0 mc. per mM) were dissolved in a convenient volume of tap water and administered orally to all patients in the postabsorptive state on the morning of the experiment. Serial blood samples of approxi mately 60 ml. were taken in heparinized syringes be ginning one hour after the administration of acetate and continued at intervals for 96 hours. Thirty five ml of plasma was then removed after centrifugation and rapidly blown into a one liter Erlenmeyer flask contain ing 525 ml. of a 4 1 mixture of dimethoxymethane methanol (6) At this point in some preliminary ex periments individual carbon 14 or tritium labelled lipides of known specific activity [2] were dissolved in one ml of petroleum ether and added to the flask. The mixture was allowed to boil momentarily by cautiously rotating the flask under a hot water tap. After cooling for fifteen minutes, with frequent swirling the mixture was filtered through an 18.5 cm. Whatman No 1 filter paper into a one liter round bottom flask with a side arm The precipitate was washed twice with an additional 30 ml. of the dimethoxymethane methanol mixture and the washings added to the lipide extract contained in the flask. The flask was placed on a constant temperature water bath maintained at 50 C and the clear yellow solution was concentrated to a small volume under re- duced pressure. Throughout this procedure oxygen free nitrogen was allowed to flow onto the surface of the liquid via the side arm. The capillary tip by which the nitrogen was delivered was not placed below the sur face of the liquid since this tended to induce excessive foaming as evaporation proceeded. Forty ml of warmed

[1] work was supported by a grant (AT(30 1) In om the Atomic Energy Commission. Additional bl obtained from institutional grants from the Fluid Fund of Yale University and the American ociety

[2] The labelled radiochemicals were obtained from the following sources a) Triolein—courtesy of Dr David Kritchevsky American Cyanamid Co., Pearl River N Y (7) b) Tripalmitin, tristearin, palmitic acid and stearic acid—Isotopes Specialties Co., Burbank, Calif c) Sterol Ester—prepared according to the procedure of Borgström (8) d) Phospholipides—prepared biosyn thetically following the administration of acetate 1-C^{14} to a subject. The phospholipides of the lipide extract of the plasma were obtained by precipitation with cold acetone and ethanolic $MgCl_2$ followed by repeated washings.

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ANNOUNCEMENT OF MEETING

The 49th Annual Meeting of the American Society for Clinical Investigation will be held in Atlantic City New Jersey on Monday, May 6, 1957, with headquarters at the Chalfonte-Haddon Hall The scientific session will begin at 9 A M at the Steel Pier Theater

TABLE I

Distribution of added radioactivity appearing in the various chromatographed fractions of plasma lipides in order of elution

Solvent system	Radioactive tracer*	Sterol esters 300 ml. 1% ether in pet. ether	Triglycerides 300 ml. 3% ether in pet. ether	Free fatty acids 225 ml 10% ether in pet. ether	Sterol 225 ml. 50% ether in pet. ether	Combined fraction Free fatty acids+Sterol eluted with 300 ml 50% ether in pet. ether	Phospholipides 225 ml. 25% methanol in ether	Recovery of radioactivity in specific fraction per cent
A	Triolein-H^3 3 120	19	2 995	79	18		3	96
	Tripalmitin 1-C^{14} 15 140	36	14 750	177	34		29	97
	Stearic acid 1 C^{14} 13 040	30	51	6 054	5 911		71	46
	Palmitic acid 1 C^{14} 11 017	37	43	5 105	5 417		84	46
	Cholesterol-4-C^{14} 20 980	24	97	14 070	5 890		228	28
	Cholesterol-4-C^{14} 20 797	36	106	13 895	6 037		185	24
B	Sterol Ester C^{14}† 8 622	8,508	143			24	47	98
	Tripalmitin 1-C^{14} 13 955	52	13 987			98	26	100
	Stearic acid 1 C^{14} 11 737	26	412			11 521	158	98
	Cholesterol-4-C^{14} 16 336	56	145			15 636	238	95
	Phospholipide-C^{14}† 1 991	28	83			170	1 715	86

* Counts per minute added to the column

† Typical examples of the separation of certain lipide fractions by chromatography with silicic acid using various solvent systems.

of 95 per cent ethanol and 0.7 ml. of 90 per cent aqueous KOH A small Soxhlet water condenser was attached to the top of each flask, a stream of nitrogen introduced via the side arm and the material refluxed for 90 minutes in a boiling water bath. During the procedure, evaporation of approximately 50 to 75 per cent of the ethanol occurred. The samples were then removed from the bath and allowed to cool. Five ml. of H_2O and twenty ml. of 95 per cent ethanol were added to each flask. The mixture was extracted three times with petroleum ether The petroleum ether washings were combined and washed once with alcoholic KOH. This ethanol wash was added to the original aqueous material remaining in the flask and the petroleum ether extracts were then discarded. The mixture was made acid to phenophthalein with 6 N H_2SO_4. Ten to twenty ml. of H_2O were added and the mixture was then extracted three times with 20-ml. aliquots of petroleum ether The combined petroleum ether extracts were washed once with 5 per cent sodium bicarbonate, three times with distilled water and then dried over anhydrous sodium sulfate. The solvent was evaporated off under nitrogen at reduced pressure and the fatty acid residue was transferred to a 25 ml. volumetric flask and brought up to volume with petroleum ether

d) *Assay* Portions of these samples were then taken in duplicate, placed in small test tubes and the solvent blown off with a stream of nitrogen. The residue was taken up in 2 ml. of 65 per cent acetone in water (v/v) and two drops of bromthymol blue added. The total fatty acids of each class of lipides was then determined by titrating the sample under nitrogen against 0 005 N KOH in a microburette calibrated in 0 001 ml. In experiments where radioactive tracers were employed, another sample was removed from the volumetric flask, transferred to a 30-ml. counting vial (Kimble K 10 Opticlear") and evaporated to dryness. To this vial was added 15 ml. of toluene containing 0 4 per cent of the phosphor 2,5 diphenyloxazole and 0.005 per cent of the wave length shifter 1 4-di [2 (5-phenyloxazolyl)]-benzene (10) The radioactivity contained in the vial was determined in a dual channel liquid scintillation counter[3] which had an efficiency of 75 per cent for carbon 14 (with an 11 1 energy acceptance window and a background of 30 cpm.) and 22 per cent for tritium. All counts were corrected for background and counted to a standard error of 2 per cent.

Part II The isolation of individual higher saturated and unsaturated fatty acids commonly found in ester linkage with the major lipide complexes

a) *Preparation of kieselguhr columns* Five pounds of "Hyflo Supercel" (Johns Manville) were thoroughly

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a) *Preparation of kieselguhr columns*. Five pounds of "Hyflo Supercel" (Johns Manville) were thoroughly

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phospholipide when higher concentrations of diethyl ether were used in the elution of "free fatty acids" and sterols from the column

The quantities of total fatty acids found in ester linkage with each of the major lipide complexes of the plasma are noted in Table II These values compare favorably with those obtained by indirect measurements (12) The fatty acids of the triglycerides in the post-prandial plasma were usually present in the highest concentration, followed by the fatty acids of the phospholipides and then those of the sterol esters

The results of the qualitative and quantitative resolution of known mixtures of some of the common straight-chain higher saturated and unsaturated fatty acids are depicted in Figures 2 and 3 Highly satisfactory separation of linolenic, linoleic, palmitic or oleic and stearic acids was accomplished by using kieselguhr column 1 cm in diameter and 120 cm in height Preliminary experiments with shorter columns led to adequate resolution of palmitic and stearic acids However, considerable overlapping was noted in the zones distinguishing linolenic from linoleic acid and linoleic from palmitic or oleic acid As noted by previous investigators (4), the presence of each double bond some distance from the carboxyl group caused an acid to act like that of a saturated acid having a chain length of two less carbons Thus, oleic, linoleic, and linolenic acids behave as and are indistinguishable from C_{16}, C_{14}, and C_{12} straight-chain saturated acids, respectively Similarly the C_{20} tetraene, arachidonic acid (an acid not available in pure form at this time) is theoretically eluted in a manner akin to that of the C_{18} triene, linolenic acid (Figures 2 and 3) or the saturated C_{12} lauric acid

TABLE II

The total fatty acids of the sterol esters, phospholipides and triglycerides

Subject	Fatty acids of sterol esters	Fatty acids of phospholipides	Fatty acids of triglycerides
	mg /100 ml	*mg /100 ml*	*mg /100 ml*
J	109	130	158
S	90 7	118	119 5
V	73 9	102 6	98 1
D	94 1	134	138

The chromatographic separation of the fatty acids of the sterol esters, the triglycerides, and the phospholipides of the plasma is depicted in Figures 4, 5, and 6 It is noted that the predominant components of all fractions are presumably linoleic, oleic, palmitic, and stearic acids The various concentrations of the individual fatty acids associated with the major lipide complexes are listed in Table III It should be stated that some titratable acidity, approximately two to four times that of the blank, occurred in the fractions eluted by 55 per cent acetone in water (v/v) Although this may represent very small quantities of either lauric (13), linolenic and arachidonic acids (4), or the products of partial oxidation of some of the higher unsaturated acids, no definite "hill and valley" elution pattern was noted The very small band that did form tended to spread widely This was probably due to the high water content of the solvent system employed under these circumstances (4, 5) Since the small quantities of these acids that were recovered precluded further accurate chemi-

TABLE III

*The distribution of the individual fatty acids associated with the major lipide complexes in the plasma of subject J**

	Linoleic acid	Oleic acid	Stearic acid	Palmitic acid	Total fatty acids of major complex	Per cent recovery of total fatty acids added to the column
	mg %	mg %	mg %	mg %	mg %	
Sterol esters	57 7	31 9	2.2	11.5	103.3	
Triglycerides	18 9	68 9	7.3	45 1	140.2	89
Phospholipides	43 1	31 2	16 1	23 1	113.5	
Per cent distribution of the individual fatty acids recovered from the total fatty acid extract						
	33.5	37 0	7.2	22.3		
Per cent distribution of the individual fatty acids present in each major complex						
Sterol esters	55 8	30 9	2 2	11 1		
Triglycerides	13 4	49 2	5.2	32.2		
Phospholipides	37 9	27.5	14 1	20 5		

* Obtained by analysis of the chromatographically separated fractions.

recovered from the kieselguhr column after the addition of this fraction Similarly 65 per cent of the titratable acidity associated with the phospholipides and 62 per cent of that found with the triglycerides were attributable to these unsaturated acids Palmitic and stearic acids on the other hand were more widely distributed in the trigly ceride and phospholipide complexes with only small quantities noted in the sterol esters

It is highly probable that both the composition and concentration of the individual fatty acids associated with each of the major lipide complexes vary considerably with the diet (15–17)

Incorporation of acetate 1 C^{14} into the total fatty acids of triglycerides the sterol esters and the phospholipides (Figure 7 Table IV)

The appearance of radioactivity in the total fatty acids of the triglycerides following the ad ministration of the C^{14}-labelled two-carbon frag ment was extremely rapid in all subjects The specific activity of these substances invariably reached a maximum at two hours declined sharply within a 24-hour period and then slowly declined during the next 72 hours A semilogarithmic plot of the data demonstrates that decay does not occur by a single exponential process and if additional points were taken over several more days the curves describing the decline of radioactivity probably could be resolved into a series of exponential rates. Thus the calculation of a composite half life for these fatty acids would not be very meaningful at this time. Undoubtedly a number of metabolic processes contribute to the disappearance of these materials from the blood stream (18)

The curve describing the specific activity of the fatty acids of the phospholipides increased more gradually to reach a plateau between 12 and 24 hours at which point it intersected the specific activity curve of the fatty acids of the triglycerides and then fell off more slowly within the next 72

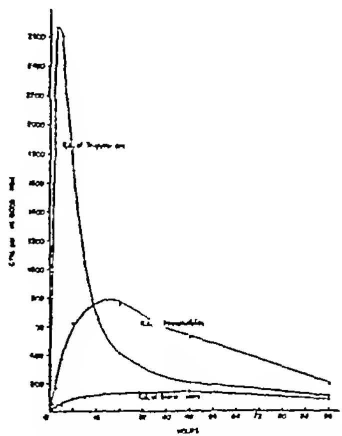

FIG. 7 THE INCORPORATION OF ACETATE 1-C^{14} INTO THE TOTAL FATTY ACIDS OF THE TRIGLYCERIDES THE PHOSPHOLIPIDES, AND THE STEROL ESTERS OF THE PLASMA OF SUBJECT J

the plasma, it seems likely that at least certain of the individual fatty acids of the phospholipides such as palmitic, stearic, and oleic acids are derived in part from or are exchanging with the fatty acids of the triglycerides The same reasoning may apply to a limited extent to the fatty acids of the sterol esters However, the lack of adequate studies concerning the relative rates of synthesis of this fraction in the mammalian liver and its presence in other tissues precludes further speculation on this point

Some information concerning the interconversion of fatty acids may be derived from an analysis of the relative specific activities of the individual acids It may be noted that despite the different concentrations of these substances in the plasma, the turnover rates of palmitic, stearic, and oleic acids within a major lipide complex are approximately comparable Furthermore, the greatest degree of radioactivity was found in the palmitic acid fraction of each group, followed then by stearic and oleic acids It would appear, from the work of Dauben, Hoerger, and Peterson (27), that palmitic acid for the most part is synthesized directly from two-carbon units and the amount derived from the process of elongation of an intermediate fatty acid such as myristic by the addition of a two-carbon fragment is exceedingly small On the other hand, Stetten and Schoenheimer (28) and Zabin (29) have indicated that a significant quantity of stearic acid is formed by direct elongation of the carbon chain of palmitic acid by two-carbon atoms Furthermore, the formation of the monounsaturated oleic acid probably occurs in a manner similar to that of the saturated acids Indeed, Anker (30) studied the relative distribution of the isotope in the various higher fatty acids after feeding myristic acid-1-C^{14} to rats and concluded that the 14 carbon atoms of myristic acid were utilized for carbon atoms 5 to 18 of oleic acid by way of palmitic and stearic acids A similar investigation employing acetate-1-C^{14} by Dauben, Hoerger, and Peterson (27) gave support to this postulate by showing that the pattern of distribution of the isotope in the degraded unsaturated C_{18} acids was identical with that of the saturated acids Additional information on the metabolic interrelationships of these acids was provided by Weinman, Chaikoff, Dauben, Gee, and Entenman (31) who found that when palmitic acid was catabolized *in vivo*, it was primarily converted to small carbon units without any appreciable formation of acids of intermediary carbon length In contrast, the catabolism of stearic acid (32), while similarly breaking down to short chain units, also gave rise to an appreciable quantity of palmitic acid The relative concentration of C^{14} in the palmitic, stearic, and oleic acid fractions isolated in this study would tend to support the occurrence of these overall reactions in man Thus, it can be assumed that palmitic acid is the major higher saturated fatty acid intermediate formed from acetate At least three pathways seem to be involved in the further metabolism of this acid First, some palmitic acid is undoubtedly deposited in the fat depots as such Second, a quantity is utilized as fuel by breaking down to two-carbon fragments which can enter the tricarboxylic acid cycle and provide a source of energy Lastly, a portion of the palmitic acid pool is converted to stearic acid Only small quantities of this C_{18} acid are found in the plasma of man under normal circumstances Presumably much of this acid is either oxidized for energy, desaturated to form oleic acid or reconverted to palmitic acid

The absence of appreciable radioactivity in the linoleic acid fraction of the major lipide complexes of the plasma lends support to the contention that this diene cannot be detectably synthesized by the mammalian liver (19, 20, 22, 28) However, it has been shown recently by Mead, Slaton, and Decker (33) that at least the carboxyl carbon of linoleic acid can be utilized to a limited extent in the formation of some of the higher saturated fatty acids in the rat The metabolic pathway involved in this reaction is not clear at this time It may be mediated via a higher unsaturated acid such as arachidonic which can be formed from a linoleate derivative (20, 22) by the addition of a two-carbon fragment or by the direct conversion of linoleic acid to short chain or more intermediate units which then can be partially utilized in the synthesis of the saturated acids This latter concept seems to be the more likely possibility

SUMMARY

Certain specific saturated and unsaturated fatty acids associated with the major lipide complexes

of the plasma of man were isolated and measured by chromatographic methods The predominant fatty acid components of the sterol esters, the phospholipides and the triglycerides were linoleic, oleic, palmitic, and stearic acids The largest quantity of unsaturated acids was noted in the sterol ester fraction The majority of the saturated fatty acids were present in the triglycerides The rates of synthesis of the total and certain of these individual higher fatty acids of man following the administration of acetate-1 C^{14} were also studied. Some of the fatty acids of the phospholipides appeared to be derived in part from those of the triglycerides The triglycerides probably represent the major vehicle for the transport of fatty acids in man as well as animals The highest concentrations of radioactivity appeared in the palmitic acid fraction of each of the major lipide complexes followed by those in the stearic and oleic acids The absence of demonstrable activity in the isolated linoleic fractions signified the lack of endogenous formation of this polyunsaturated fatty acid in man

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Some information concerning the interconversion of fatty acids may be derived from an analysis of the relative specific activities of the individual acids It may be noted that despite the different concentrations of these substances in the plasma, the turnover rates of palmitic, stearic, and oleic acids within a major lipide complex are approximately comparable Furthermore, the greatest degree of radioactivity was found in the palmitic acid fraction of each group, followed then by stearic and oleic acids It would appear, from the work of Dauben, Hoerger, and Peterson (27), that palmitic acid for the most part is synthesized directly from two-carbon units and the amount derived from the process of elongation of an intermediate fatty acid such as myristic by the addi- of a two-carbon fragment is exceedingly small ne other hand, Stetten and Schoenheimer nd Zabin (29) have indicated that a signifi- quantity of stearic acid is formed by direct ngation of the carbon chain of palmitic acid by wo-carbon atoms Furthermore, the formation of the monounsaturated oleic acid probably occurs in a manner similar to that of the saturated acids Indeed, Anker (30) studied the relative distribution of the isotope in the various higher fatty acids after feeding myristic acid-1-C^{14} to rats and concluded that the 14 carbon atoms of myristic acid were utilized for carbon atoms 5 to 18 of oleic acid by way of palmitic and stearic acids A similar investigation employing acetate-1-C^{14} by Dauben, Hoerger, and Peterson (27) gave support to this postulate by showing that the pattern of distribution of the isotope in the degraded unsaturated C_{18} acids was identical with that of the saturated acids Additional information on the metabolic interrelationships of these acids was provided by Weinman, Chaikoff, Dauben, Gee, and Entenman (31) who found that when palmitic acid was catabolized *in vivo*, it was primarily converted to small carbon units without any appreciable formation of acids of intermediary carbon length In contrast, the catabolism of stearic acid (32), while similarly breaking down to short chain units, also gave rise to an appreciable quantity of palmitic acid The relative concentration of C^{14} in the palmitic, stearic, and oleic acid fractions isolated in this study would tend to support the occurrence of these overall reactions in man Thus, it can be assumed that palmitic acid is the major higher saturated fatty acid intermediate formed from acetate At least three pathways seem to be involved in the further metabolism of this acid First, some palmitic acid is undoubtedly deposited in the fat depots as such Second, a quantity is utilized as fuel by breaking down to two-carbon fragments which can enter the tricarboxylic acid cycle and provide a source of energy Lastly, a portion of the palmitic acid pool is converted to stearic acid Only small quantities of this C_{18} acid are found in the plasma of man under normal circumstances Presumably much of this acid is either oxidized for energy, desaturated to form oleic acid or reconverted to palmitic acid

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RAPID AND SLOW COMPONENTS OF THE CIRCULATION IN THE HUMAN FOREARM[1]

By EDWARD D FREIS HAROLD W SCHNAPER, AND LAWRENCE S LILIENFIELD[2]

(From the Cardiovascular Research Laboratory Georgetown University School of Medicine and the Veterans Administration Hospital Washington D C)

(Submitted for publication April 20 1956 accepted October 1 1956)

The present study is concerned with the manner in which a segment of arterial blood flows through the vessels of the human forearm Specifically it is directed toward answering the question whether blood flows as a unit or is distributed into channels with varying rates of flow The experimental procedure employed has as its basis the fact that the forearm circulation is small in comparison to that of the total body

Volume dilution methods as commonly employed for measuring either cardiac output or total blood volume require a dose of dye or other labelling material sufficiently large to produce significant concentrations in the general circulation. In the cardiac output method only the upslope, peak and first portion of the downslope may be obtained before distortion occurs due to contamination with recirculating labelled material

In the forearm, however only a small quantity of dye or other labelling substances injected into the brachial artery produces easily measurable concentrations in the effluent veins The portion of this small dose of dye which escapes into the general circulation becomes so well diluted that its concentration in vessels elsewhere in the body cannot be detected by ordinary spectrophotometric methods Hence, the washout of the dyed segment of forearm arterial blood can be studied without contamination or distortion by significant amounts of recirculating injectate

The purpose of this investigation was not to measure absolute blood flow of the forearm and hand The validity of indicator dilution methods has not been established for making such measurement in a peripheral area which has multiple venous drainage (1) Rather the concern was with the characteristics of the uncontaminated down slope of the labelled blood in the area drained by the particular effluent vein being sampled

MATERIALS AND METHODS

The subjects either were normal or patients on the hospital wards They were all young or early middle-aged males. All patients were afebrile and ambulatory for at least one week prior to serving as subjects for these experiments. None were suffering from diseases of the cardiovascular system.

The method was similar to that described in a previous communication (2) except that the amount of dye injected was smaller One-fourth ml. of a 0.5 per cent solution of the blue dye T 1824 was diluted in a mixture containing 3.5 ml of deuterium oxide in saline and 0.2 ml of 5 per cent sodium thiocyanate in distilled water Three ml. of this mixture was injected into the brachial artery through a 20 gauge needle attached to a three-way stopcock. Thiocyanate and deuterium oxide were added to the mixture in order to study the time-concentration curves of these permeable substances, as will be described in future reports.

Immediately following the injection sampling was begun through a 17 gauge "thin walled" needle threaded well into a large antecubital vein. Whenever possible a vein was chosen which appeared to drain the deep as well as the superficial structures Samples were collected in the manner described previously (2) usually at intervals of 2 seconds then 20 second intervals to 4 minutes then 5 6 8, 10 12 and 15 minutes respectively A sample was withdrawn from a vein in the opposite arm 3 to 5 minutes after the injection in order to rule out the possibility of significant amounts of recirculating labelled material.

Several precautions were necessary to insure valid results. The needle in the vein was directed against the stream of flow and the bevel so placed as to provide good outflow and adequate samples. Scanty samples resulted when the bevel was against the side of the vein, producing distorted curves probably due to delay and mixing in the needle and collecting catheter In cases in which an adequate outflow could not be obtained (less than 0.5 ml. of blood per second) the results were discarded. The arterial needle also must be well placed, permitting unobstructed injection of the tracer materials

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[2] Helen H Millenson Memorial Fellow Metropolitan Heart Guild, Washington, D C.

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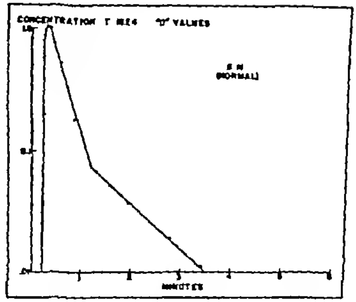
which the dye appeared and rapidly increased in concentration forming a steep ascending phase lasting generally a few seconds The curve fell away at first rapidly and then more slowly The transition between the early and late phases of the downslope either was abrupt or connected by a short transition zone. The two phases of the downslope both were exponential forming straight lines when plotted on semi log paper In 10 cases the early steep downslope and later shallower downslope were connected directly without an intervening transition zone (Figure 1a) In the remaining 15 subjects a transition zone which was intermediate in gradient connected the early and late phases of the downslope (Figure 1b)

Although the majority of the curves showed clearly delineated biphasic downslopes there were some variations from the type of curves illustrated. The variations seen included irregularity of upslope, "sawtooth' or double peaks and small, late, third phase which was either shallower or steeper than the preceding phase. These variations usually were observed in the cases in which small samples were obtained presumably because of poor venous blood flow In the calculations which follow, these variations were rounded off" because they would be difficult to subject to mathematical treatment and were not representative of the majority of the curves

The biphasic downslopes were not due to differences in velocity of blood flow through the hand as compared with the forearm In 9 subjects the hand was excluded by inflating a cuff on the wrist to pressures 100 mm Hg above systolic pressure (Table I) Typical biphasic downslopes were seen in 8 of these cases In two subjects (A R. and E P Table I) the injection was made into the radial artery and sampling was carried out through a vein in the wrist. Multiphasic down slopes were obtained in both instances

Analysis of the biphasic downslope into components with different flows and volumes

Reasons are presented in the discussion for believing that the biphasic downslopes represent a rapid flow component producing the early steep downslope and a slower component producing the later shallow downslope. It was desirable to analyze these component parts quantitatively in terms



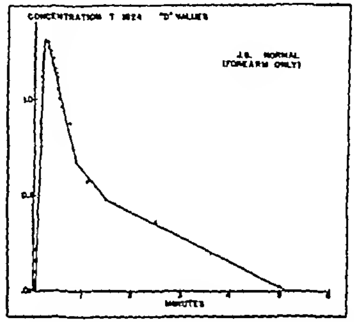


FIG. 1 GRAPHS ILLUSTRATING TYPICAL BIPHASIC TIME CONCENTRATION WASHOUT SLOPES IN THE HUMAN FOREARM

Figure 1a (above) illustrates a simple biphasic exponential curve. Figure 1b (below) shows a biphasic curve with transition zone.

of flow and volume Absolute measurements of flow were not possible because the dye is not always distributed uniformly in the forearm (1) However the relative flows and relative volumes in the biphasic system could be determined using only the mean circulation times

The solution was based on the well known relationship that vascular volume from the point of injection to the point of sampling is equal to blood flow multiplied by the mean circulation time Thus v_1 the vascular volume of the rapid component, was equal to f_1 (the flow in this component) times t_1 (its mean circulation) Similarly v_2 (the volume of the slow component) equalled f_2 times t_2 and V (the total vascular volume of the seg

and the injection must be accomplished evenly, gently and rather slowly (0.5 to 1 0 ml per second) in order to avoid arterial spasm Arterial spasm was recognized by a sudden temporary decrease in outflow from the venous drainage needle. When this occurred the results were discarded.

The dye concentrations in the plasma were determined spectrophotometrically using 1-ml cuvettes It usually was necessary to dilute the samples containing the peak dye concentrations (and the plasma blank) with saline in order to obtain accurate readings Occasional hemolyzed samples either were discarded or were read after precipitating the proteins of the sample and plasma blank with acetone. The resulting dye density values were plotted on semi-log paper Chromium[51] labelled red cells were prepared and the radioactivity of the samples was determined by the method of Sterling and Gray (3)

Mean circulation time was calculated as follows each concentration per unit time was multiplied by its respective time from the midpoint of injection The sum of these products then was divided by the sum of the concentrations The volume of the collecting system (needle, stopcock and collecting catheter) was measured and appropriate correction made for delay in the catheter

RESULTS

Uncontaminated curves were obtained in 28 subjects, in 25 with the dye T-1824 and in 3 with Cr^{51} labelled red cells (Table I) A monophasic downslope was obtained in only 3 of these cases In the remaining 22 patients receiving the dye and in the 3 cases receiving Cr^{51} labelled red cells the downslopes were biphasic Representative biphasic curves are illustrated in Figure 1 After injection there was a brief, latent period following

TABLE I

Mean circulation times and ratios of flows and volumes in the forearm circulation

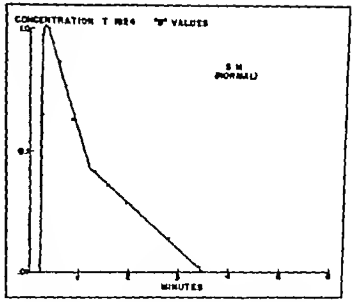
Case	Age	Diagnosis	Type of curve*	T	t_1	t_2	f_1/f_2	v_1/v_2
J C	27	U R.I	M	72				
C F	28	Toxic hepatitis, conval 9 weeks	B + tr	35	22	95	4 6	1 1
C H	32	U R I	B − tr	70	28	105	1 0	0 2
C T	30	Atypical pneumonia	B − tr	79	56	283	5 1	1 4
H F	30	Lobar pneumonia	B + tr	27	25	66	19 5	7 5
J G	33	Psychoneurosis	M	29				
G P	43	Peptic ulcer	B + tr	125	50	178	0 7	0 2
J O	31	Diabetes mellitus	B + tr	51	18	102	1 5	0 25
R T	31	Idiopathic pericarditis	B − tr	61	55	210	26 0	6 8
A G	28	Early cirrhosis	B + tr	63	21	128	1 5	0 25
H W	27	Psychoneurosis	B + tr	38	22	140	6 4	1 0
M W	30	Peptic ulcer (Cephalic vein)	B + tr	56	30	91	1 3	0 4
		(Median vein)	B + tr	65	35	101	1 2	0 4
S M	48	Bronchial asthma	B − tr	51	41	119	6 8	2 3
Hand only								
A R.	31	U R I	B + tr	41	31	115	7 4	2 0
E P	40	Peptic ulcer	B + tr	67	40	150	3 1	0 8
Forearm only								
J G	36	Erythema multiforme	B + tr	35	23	139	8 7	1 4
E C	45	Bursitis	M	114				
J H	33	Peptic ulcer	B + tr	102	49	239	2 6	0 5
W C	32	Urticaria	B − tr	32	17	86	2 3	0 5
J E	38	No disease found	B − tr	70	64	349	47 0	8 5
W J	27	Diabetes mellitus	B − tr	110	21	176	0 7	0 1
A M	30	Psychoneurosis	B − tr	90	35	136	0 8	0 2
J M	28	Latent syphilis	B − tr	39	26	105	5 0	1 25
C. E	24	No disease found	B + tr	61	22	99	0 8	0 2
Cr^{51} RBC								
E W	34	Peptic ulcer	B − tr	36	32	112	19 0	3 8
B H	54	Essential hypertension, mild	B + tr	27	13	116	6 4	0 7
F M	48	Essential hypertension, mild	B + tr	17	13	64	11 8	2 4
		Mean and S D		59 1 ±29 0	31 4 ±14 1	146 0 ±72 4	7 6 ±10 5	1 8 ±2 4
		Median					5 1	1 0

* M = Monophasic downslope B + tr = Biphasic downslope with transition zone, B − tr = Biphasic downslope without transition zone.

which the dye appeared and rapidly increased in concentration forming a steep ascending phase lasting generally a few seconds The curve fell away at first rapidly and then more slowly The transition between the early and late phases of the downslope either was abrupt or connected by a short transition zone. The two phases of the downslope both were exponential forming straight lines when plotted on semi log paper In 10 cases the early steep downslope and later shallower downslope were connected directly without an intervening transition zone (Figure 1a) In the remaining 15 subjects a transition zone which was intermediate in gradient connected the early and late phases of the downslope (Figure 1b)

Although the majority of the curves showed clearly delineated biphasic downslopes there were some variations from the type of curves illustrated The variations seen included irregularity of upslope, "sawtooth' or double peaks and small late third phase which was either shallower or steeper than the preceding phase These variations usually were observed in the cases in which small samples were obtained presumably because of poor venous blood flow In the calculations which follow these variations were 'rounded off" because they would be difficult to subject to mathematical treatment and were not representative of the majority of the curves

The biphasic downslopes were not due to differences in velocity of blood flow through the hand as compared with the forearm In 9 subjects the hand was excluded by inflating a cuff on the wrist to pressures 100 mm Hg above systolic pressure (Table I) Typical biphasic downslopes were seen in 8 of these cases In two subjects (A R. and E. P Table I) the injection was made into the radial artery and sampling was carried out through a vein in the wrist. Multiphasic downslopes were obtained in both instances.



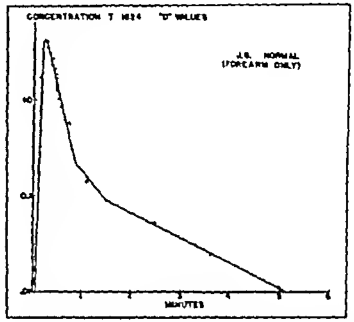


FIG. 1 GRAPHS ILLUSTRATING TYPICAL BIPHASIC TIME CONCENTRATION WASHOUT SLOPES IN THE HUMAN FOREARM

Figure 1a (above) illustrates a simple biphasic exponential curve. Figure 1b (below) shows a biphasic curve with transition zone.

Analysis of the biphasic downslope into components with different flows and volumes

Reasons are presented in the discussion for believing that the biphasic downslopes represent a rapid flow component producing the early steep downslope and a slower component producing the later shallow downslope. It was desirable to analyze these component parts quantitatively in terms of flow and volume. Absolute measurements of flow were not possible because the dye is not always distributed uniformly in the forearm (1) However the relative flows and relative volumes in the biphasic system could be determined using only the mean circulation times

The solution was based on the well known relationship that vascular volume from the point of injection to the point of sampling is equal to blood flow multiplied by the mean circulation time Thus v_1 the vascular volume of the rapid component, was equal to f_1 (the flow in this component) times t_1 (its mean circulation) Similarly v_2 (the volume of the slow component) equalled f_2 times t_2 and V (the total vascular volume of the seg

DISCUSSION

Several hypotheses can be advanced to explain the observed biphasic system The first to be considered is differences in path lengths According to Wiggers (4) the velocity of blood flow in the various subdivisions of the vascular tree varies inversely with the cross sectional area, decreasing gradually in the arteries from an aortic velocity of 18 cm per second, slowing abruptly in the arterioles and capillaries where velocities of approximately 5 mm per second exist, and increasing again in the large veins to 5 or more cm per second Assuming the longest probable path length in the forearm to be 80 cm for both arterial and larger venous segments, 4 cm for the arteriole, 2 cm for the capillary, 4 cm for venule and 10 cm for the small venous segments, and employing conservative estimates of velocity in these segments, transit through the longest pathway would be less than 30 seconds This fails to agree with the mean circulation times observed in the present experiments, since mean t_2 was 141 seconds or approximately 5 times the duration required for blood to flow through the longest pathway on the basis of presently assumed mean velocities (4)

The actual distribution of path lengths in the forearm would be expected to follow a typical population or "bell-shaped" distribution curve If the velocities of flow were uniform in each subdivision throughout the forearm and difference in path lengths was the only variable, the downslope would be monophasic, the ascending limb representing the shortest pathways, the peak the numerically greatest path lengths and the downslope the longest pathways In order to explain the presently observed biphasic system two distinct populations of pathways are assumed, both being present in the hand alone and in the forearm alone.

If path length was the only variable, and since t_2 averaged more than 4 times t_1, it also is assumed that the 2 populations of path lengths differed from each other by a factor of 4 On the basis of presently accepted circulatory velocities (4) the capillaries composing the slow compartment would average about 100 cm in length a figure which seems to be incredibly large

The two compartment system also might be explained on the basis of differences in blood velocity through the principal tissues of the forearm, namely, muscle and skin Blood velocity through the skin alone can be estimated by observing directly the passage of appropriate doses of fluorescein or T-1824 in the forearm following brachial arterial injection (5) Peak dye concentrations occur within 10 to 30 seconds after the dye is injected, although traces can be seen in heavily stained areas for longer periods These studies indicate that the major portion of blood flow to the skin travels at velocities consistent with t_1 and not t_2 Thus, if the slow component is limited to one of the major tissue subdivisions of the forearm, it probably resides in the muscles

Data to be presented later do not support this explanation Local muscular exercise to the point of exhaustion was carried out by having the subject repeatedly squeeze a hand dynamometer Dye curves following such local exercise revealed proportionate reductions of both t_1 and t_2 compared with the controls Since muscle and not skin was affected predominantly by such exercise, and if muscle were the site of the slow component, there should have been a disproportionate reduction of t_2 In addition, in the untreated subject biphasic curves were observed in the hand alone, which contains little muscle, and in forearm alone (exclusive of the hand and wrist), which contains predominantly muscular tissue

Renkin, utilizing a different technique, observed similar biphasic blood flow patterns in the perfused hindleg of the cat (6) The relative volumes of the two components were uninfluenced by removing the skin The proportion of bone was too small to account by itself for one compartment and Renkin concluded that both compartments are present in skeletal muscle These various observations make it appear unlikely that the two-phase velocity system is produced by differences in blood velocity in different tissues

Barcroft presents evidence pointing toward a double circulation in the human forearm and calf (7) He believes that one is under neurogenic control, the other under the influence of local metabolites The measurements were determined on mean blood flow and its response to various stimuli

Whereas in the normal subjects flow in the rapid component usually predominated, the vas-

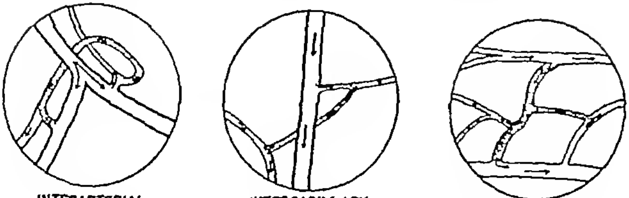


FIG. 6. TYPICAL ANASTOMOTIC CONNECTIONS BETWEEN ARTERIOLES, CAPILLARIES AND VENULES AS OBSERVED IN THE HAMSTER CHEEK POUCH PREPARATION

Arrows indicate the direction of flow Arrows with heads pointing in opposite directions and stipled sections indicate anastomotic vessels with sluggish and reversible flow

cular volumes of the two components were of the same magnitude. Thus, nearly half and in many of the subjects more than half of the vascular volume of the forearm contained slowly circulating blood As has been outlined above the evidence pointed away from localizing rapid blood flow to one type of tissue and sluggish flow to another and suggested that the two flow systems existed side by side in both skin and muscle

In seeking for the location of this large vascular component we have observed the circulation in the hamster cheek pouch according to the method of Lutz and Fulton (8) Using 100 to 300 times magnifications it was apparent immediately that the small vessel circulation has a biphasic pattern of blood velocity In every field vessels of the same order were seen with widely varying rates of blood flow (Figure 6)

The mechanism for the variation of velocity was apparent. The circulation was highly anastomotic with many interarteriolar intercapillary and intervenular connections A similar anastomotic circulation has been described in the bat's wing (9 10) in the subcutaneous tissue of mice (11) in voluntary muscle (12) and in intestinal wall (13) Since pressures at either end of the anastomosis were similar flow was sluggish in the anastomosis Indeed it sometimes would stagnate surge first one way and then the other and occasionally reverse.

Flow through this network of small vessels also was highly dynamic. A capillary or venule appearing to contain the rapid or main line flow would suddenly change into a sluggish channel Similarly slow moving flow in an anastomotic vessel would for short periods become rapid moving sometimes in one direction, at other times in the opposite direction. Thus not only do the rapid and slow moving circulations exist side by side but some of the small vessels change their role from time to time, now carrying rapid and at another time sluggish flow In a system composed of anastomotic channels relatively minor shifts in pressure could easily change the pattern of blood flow The factors controlling such pressure fluctuations are not completely understood although contraction of arterioles and precapillary sphincters probably play a part. Although these direct observations on the microcirculation of other preparations do not prove that the slow component of the forearm circulation in man is due entirely to retarded flow in anastomotic vessels they provide a reasonable hypothesis for further study

An alternative method of calculating the relative flows and volumes would be to treat the two curves as if they were occurring *synchronously* rather than successively In this situation the late downslope is extrapolated back to the peak of the primary curve (since the two curves are assumed to be synchronous) and the values so obtained subtracted from the early downslope This will produce a decrease in the ratios f_1/f_2 and v_1/v_2 *i e* f_2 and v_2 *will become larger relative to* f_1 and v_1 (Table II) This method of analysis seems to lack validity for the following reasons In the present experiments the observations began at the instant of first injection of the tracer material which was confined to the intravascular space. The tracer entered as a bolus and was preceded and followed by undyed blood In essence it

THE GLUCOSE METABOLISM OF PATIENTS WITH MALIGNANT DISEASE AND OF NORMAL SUBJECTS AS STUDIED BY MEANS OF AN INTRAVENOUS GLUCOSE TOLERANCE TEST [1, 2]

By PAUL A. MARKS AND JONATHAN S BISHOP [3]

(From the Medical Services of The Francis Delafield Hospital and The Presbyterian Hospital Department of Medicine, Columbia University College of Physicians and Surgeons, New York City, N Y)

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Since the observations of Freund (2) in 1885 of spontaneous hyperglycemia in patients with malignant disease, there has been speculation that an alteration in glucose metabolism is associated with neoplasia in man (3–18) There have been, however, relatively few studies designed specifically to test this possibility Rohdenburg, Bernhard, and Krehbiel (5) and Edwards (6), with the advent of the oral glucose tolerance test, reported that a decrease in carbohydrate tolerance was a uniform finding in patients with cancer Subsequent studies (7–18) did not confirm these reports, though several workers have found a high incidence of an abnormal glucose tolerance test in subjects with various types of neoplasms These observations have been inconclusive with regard to establishing a relationship between the presence of uncomplicated neoplastic disease and an altered carbohydrate metabolism A primary reason for the inconclusive nature of these studies is their lack of control of various factors known to decrease glucose tolerance Certain of these factors, *e g*, inadequate dietary intake, fever, infection, hepatic dysfunction and prolonged bed rest, are frequently present in patients with malignant disease

This study was undertaken in a group of carefully selected patients with chronic leukemia, lymphoma, and clinically early epithelial neoplasms, and subjects without cancer, in an effort to determine whether a defect in glucose metabolism is associated with malignant tumors It was found that, as a group, the patients with neoplastic disease had a significant decrease in the rate of disappearance of glucose from the blood following the intravenous injection of glucose Carbohydrate metabolism was evaluated further in these subjects by calculation of the net rate of disappearance of glucose from the blood and the volume of distribution of glucose and by determination of the changes in serum inorganic phosphate and potassium concentrations during the glucose tolerance tests

METHODS

Subjects studied

The control group consisted of 19 individuals without neoplastic disease, 7 males and 12 females, ranging in age from 29 to 65 years The neoplastic group included 36 individuals, 14 males and 22 females ranging in age from 36 to 70 years There was no significant difference between the control and neoplastic groups with respect to age or body weight Subjects 20 through 33 had clinically localized carcinoma, which was in all instances considered amenable to curative therapy These individuals had no systemic symptoms Carcinoma was suspected on the basis of a finding of a lump in the breast on physical examination, a positive Papanicolaou smear on routine cervical examination, or an abnormal shadow on routine chest x-ray Subjects 34 through 54 had lymphoma or chronic leukemia The diagnosis in each individual was established histologically (Tables I and II) All subjects were studied preoperatively, or prior to chemotherapy or radiotherapy

The criteria for selection of individuals for study in both the control and patient group included 1) no family history of diabetes mellitus, 2) full ambulation, 3) no weight change for at least six months prior to the study, 4) no obesity, 5) no evidence or history of any disorder known to affect carbohydrate metabolism, 6) no fever 7) normal serum concentrations of sodium, potassium, chloride, carbon dioxide non protein nitrogen, alkaline phosphatase, cholesterol and cholesterol ester, and a normal cephalin flocculation and 8) an adequate dietary intake.

[1] A portion of the present data has been published previously in abstract form (1)

[2] This work was supported in part by a grant from The National Cancer Institute, U S Public Health Service.

[3] Fellow of the American Cancer Society

TABLE I

Intravenous glucose tolerance test in control subjects

Subject No.*	Fasting glucose†	Glucose concentration (mgm. %) (Time in minutes) 8	16	24	32	40	48	56	64	Disappearance rate constant (%/min.) Total	Increment	Mean glucose concentration‡ (mgm. %) Total	Increment	Net glucose disappearance‡ (mgm. %/min.) Total	Increment
1	79	266	231	200	175	156	141	129	119	1.51	2 81	235	139	3 68	3 91
2	64	219	192	185	153	115	96	87	80	2 30	4 62	223	112	5 12	5 19
3	88	288	240	188	164	124	92	76	73	2.89	4.36	180	111	5.20	4 85
4	80	304	244	200	176	148	120	92	86	2 20	3 15	209	120	4 60	4 50
5	100–98	263	222	202	179	164	151	140	125	1 31	3 10	200	89	2 62	2 76
6	86–88	284	198	135	110	87	64	60	59	3 47	7 96	132	66	4.58	5.25
7	75–76	198	191	164	144	120	106	96	89	1 78	3.22	158	80	2 81	2 58
8	68–66	250	216	175	148	125	109	98	77	2 30	4 08	183	103	4.21	4 20
9	88–85	188	176	152	131	122	108	104	99	1.56	3 96	206	81	3.21	3 21
10	92–90	275	243	198	176	161	147	134	122	1 51	3 15	209	105	3 16	3 31
11	83–83	175	156	138	128	117	110	95	83	1.33	3.22	160	62	2 12	2 00
12	89–94	264	208	176	145	121	110	92	86	2 16	5 10	185	76	5 00	3 88
13	82–87	188	156	122	104	92	92	90	87	2 01	6 42	143	43	3 73	3.58
14	73–72	180	172	155	141	128	117	110	102	1.31	2 78	151	74	2 36	2 49
15	83–81	231	192	147	123	108	97	78	70	2 08	6.30	208	67	4 83	4.52
16	81–70	240	230	193	174	158	137	134	110	1 59	2 90	199	113	3 16	3 28
17	92–88	258	212	188	175	160	140	132	111	1 61	2 78	187	104	2 98	2 86
18	78–82	210	186	160	145	131	114	94	80	1.58	3 15	165	80	2.74	2 62
19	84–85	250	168	142	115	104	90	87	82	3 15	6.03	142	73	4 88	4 03
Average	83	238	202	164	148	129	113	101	92	1 97	4.20	180	89	3 74	3 68
S D	8	38	28	26	23	23	22	22	18	62	1 52	29	24	1 04	0 93

* Diagnoses Cases 1–5 9 11–13 16–19 Volunteers Cases 6, 7 10 14 15 Fibroadenoma of breast Case 8 Cervical erosion

† Where single value for fasting glucose is given only one fasting blood sample was obtained

‡ Refer to text for definition of these terms and methods of calculating their values.

Procedures

All subjects were placed on a diet containing at least 225 grams of carbohydrate and 2400 calories daily for two weeks prior to study Each individual, having fasted overnight for 14 hours was kept completely at rest in an air conditioned room (temperature 78 to 81 F) for one-half hour prior to and during the test. An indwelling Cournand type needle was placed in an antecubital vein. After two fasting blood samples had been obtained over a period of approximately 20 minutes a 30 per cent glucose solution in distilled water was injected into another vein. In control subjects 1 through 11 and patients 20 through 27 and 34 through 42, twenty five grams of glucose was administered from a syringe over a four-minute period. In all other individuals, the glucose was administered using a Bowman constant infusion pump over a three to five-minute period. This permitted a more accurate determination of the amount of glucose delivered (Table VI)

Venous blood specimens were withdrawn without stasis into heparinized syringes at eight minutes following the start of the glucose injection and subsequently every eight minutes for 64 minutes. Glucose concentration and, when performed, serum inorganic phosphate and serum potassium concentrations were determined in duplicate on each blood sample. The blood specimens were immediately iced and protein-free filtrates for the glucose analyses were promptly prepared at the bedside. Urine was collected during the 75 minutes following the injection of glucose for the determination of urinary glucose excretion during the test.

Ten patients had 2 or 3 repeat glucose tolerance tests performed (Table III) Six of these subjects were maintained on a constant carbohydrate and caloric intake on the metabolic ward during the interval between the studies. The remaining four individuals followed the prescribed diet on an outpatient basis during the period of observation.

Glucose was determined in duplicate by the Nelson modification of the Somogyi method (19 20) Serum inorganic phosphorus determinations were made according to the method of Taussky and Shorr (21) Serum potassiums were determined using a flame photometer with an internal standard.

Analysis of glucose tolerance curves

Methods of analysis of the rapid intravenous glucose tolerance test have varied. In the present study each glucose tolerance curve was graphically evaluated both by the method suggested by Conard Franckson, Bastenie, Kestens, and Kovacs (22) and by the technique employed by Amatuzio Stutzman, Vanderbilt, and Nesbitt (23) These two methods were chosen because (a) they provide methods which facilitate analyzing the glucose tolerance curve in terms of a single constant, (b) a large amount of normal data is available in the literature for these techniques, and (c) in the present study by means of statistical analyses which will be presented below

TABLE IV

Analysis of variance of indices of repeat glucose tolerance tests in ten patients with neoplastic disease

	Degrees of freedom	Total index	Increment index
		Sum of squares	Sum of squares
Between individuals	9	09951	57087
Within individuals	10	00263	02477
Total	19	10215	59564
F*		41 92	25 61

$$^{*}F = \frac{\text{Sum of Squares Between Individuals}}{\text{Sum of Squares Within Individuals}} \times \frac{10}{9}$$

For the number of degrees of freedom present in this table, an F value greater than 4 95 would give a probability less than 0 01

disappearance, *i e*, the per cent of glucose disappearing from the blood per minute. In an effort to evaluate the net amount of glucose disappearing from the blood, an estimate was made of the mean net rate of disappearance of glucose, *i e*, the milligrams of glucose disappearing per unit volume of blood per minute. The term net is used to indicate that this value represents the resultant of those reactions removing glucose from the blood and those reactions delivering glucose to the blood. In order to obtain the mean net rate of disappearance of 'total glucose" from the blood per minute (GD_b), the average "total glucose" concentration was multiplied by the rate constant K_b The mean net rate of disappearance of "increment glucose" from the blood (GD_a) was derived by multiplying K_a by the mean glucose concentration in excess of the fasting level[5] All values for net rate of disappearance of glucose, in subjects receiving other than 25 gm of glucose, were corrected to this dose. The value obtained for mean net rate of disappearance of glucose is

[5] The value for the average "total glucose' concentration was obtained by the equation

$$\overline{G}_b = \frac{G_o(1 - e^{-K_b t})}{t K_b},$$

where $\overline{G}_b$ is the average 'total glucose' concentration in mgm. per 100 cc., G_o is the total blood glucose concentration in mgm. per 100 cc. at zero time, t is the time interval in minutes during which the fractional rate of total glucose disappearance is constant.

The value for the average 'increment glucose' concentration was obtained by the equation

$$\overline{G}_a = \frac{(G_o - G_f)(1 - e^{-K_a t})}{t K_a}$$

where $\overline{G}_a$ is the average "increment glucose" concentration in mgm. per 100 cc., G_f is the fasting blood glucose concentration in mgm. per 100 cc. t is the time interval in minutes during which the fractional rate of "increment glucose" disappearance is constant.

defined by the method employed in its calculation It is used in this study only for the purpose of comparing the control and neoplastic groups with respect to this parameter The value for GD_b would be expected to be equal to the value for GD_a if both a plot of the log of the "total glucose" concentration against time and a plot of the log of the "increment glucose' concentration against time represent good approximations of a straight line.

Estimation of volume of distribution of glucose An estimate of the volume of distribution of glucose (EVG) was obtained in those subjects in whom the glucose was given using a constant infusion pump to permit an accurate determination of the volume of glucose solution injected. The EVG was calculated by dividing the amount of glucose injected (mgm) by the glucose concentration in excess of the fasting value (mgm per 100 cc.) at the end of injection This glucose concentration was determined by extrapolating to the end of the injection period the linear plot of the log of the glucose concentration in excess of the fasting level between 16 and 56 minutes following the injections The values for blood sugar concentration prior to 16 minutes generally fell above this line and were not employed in this graphic analysis to reduce the error due to mixing It is recognized that this calculation is not free from objection, particularly when applied to metabolizable substances Nevertheless, it is assumed that these errors will be similar in both control and neoplastic subjects

RESULTS

The data obtained from intravenous glucose tolerance tests in 19 control subjects and 36 patients with neoplastic diseases are presented in Tables I and II The diagnosis of each subject is indicated in these tables

Fractional rate constant of glucose disappearance

The fractional rate of "total glucose" disappearance (K_b) for the control group was 1.97 ±

TABLE V

Analysis of variance comparing the glucose tolerance tests in control subjects and patients with neoplastic disease

	Degrees of freedom	Total blood sugar index	Increment blood sugar index
		Sum of squares	Sum of squares
Between groups	1	64261	10 38503
Within groups	54	1 66644	27 77842
Total	55	2 30905	38 16345
F*		20 82	20 18

$$^{*}F = \frac{\text{Sum of Squares Between Groups}}{\text{Sum of Squares Within Groups}} \times \frac{54}{1}$$

For the number of degrees of freedom present in this table, an F value greater than 7 12 would give a probability less than 0 01

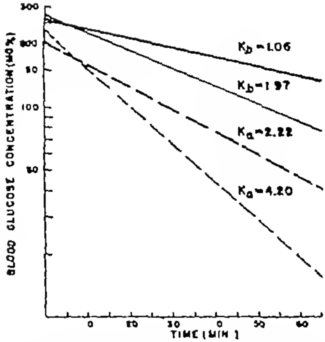

FIG. 1 THE AVERAGE CURVES OF TOTAL BLOOD GLUCOSE CONCENTRATION AGAINST TIME ARE PLOTTED FOR THE CONTROL (LIGHT SOLID LINE) AND PATIENT (HEAVY SOLID LINE) GROUPS AND THE AVERAGE CURVES OF THE BLOOD SUGAR CONCENTRATION IN EXCESS OF THE FASTING LEVEL AGAINST TIME ARE PLOTTED FOR THE CONTROL (LIGHT BROKEN LINE) AND PATIENT (HEAVY BROKEN LINE) GROUPS

The fractional rate of disappearance of "total glucose" (K_b) and the fractional rate of disappearance of "increment glucose" (K_a) are indicated in per cent per minute.

0.62 per cent per minute.* The K_b for the neoplastic group was 1 06 ± 0.33 per cent per minute. The fractional rate of "increment glucose" disappearance (K_a) in the control group averaged 4.20 ± 1 52 per cent per minute The neoplastic group had a K_a of 2.22 ± 0.85 per cent per minute. The difference between the means for both K_a and K_b were statistically significant † (Figure 1) Examination of Tables I and II will reveal that K_b in five subjects with neoplastic disease and K_a in six of these patients fell within the range of values for the control series

The data for the control subjects are in general agreement with previous results obtained using the type of intravenous glucose tolerance test employed in this study In 60 normal subjects reported by Lozner Winkler, Taylor and Peters (26) the K_b was 1.96 ± 0.30 per cent per minute and the K_a was 3 57 ± 0 37 per cent per minute. Amatuzio Stutzman Vanderbilt and Nesbitt (23) reported data on 70 control individuals with a K_b of 1 34 ± 0.27 per cent per minute and a K_a of 3.71 ± 0 40 per cent per minute. An average K_b of 1 52 ± 0.23 per cent per minute has been observed in 20 normal subjects (22) In 20 normal persons, Duncan (24) found a K_b of 1 37 ± 0.22 per cent per minute and a K_a of 3 68 ± 0 40 per cent per minute

Blood sugar values

The fasting blood sugar for the control series was 83 ± 8 mgm per cent and for the neoplastic group 89 ± 13 mgm per cent (Tables I and II) The difference between these means was not statistically significant. Four patients with neoplastic disease did have fasting blood sugars in excess of 100 mgm. per cent, which was the upper value for the range of fasting blood sugar levels in the control group

The average blood sugar values for the control and neoplastic groups at 8 and 16 minutes following glucose administration were not significantly different. The mean blood sugar concentrations at subsequent sampling times *i.e.*, 24 32, 40 56 and 64 minutes following glucose administration were significantly higher in the patients with neoplastic disease than in the control individuals (Tables I and II)

Urinary glucose excretion during the 75 minutes following the intravenous administration of glucose was determined in 14 control individuals and 22 patients Urinary glucose excretion amounted to 0.8 ± 0.3 gm during the 75-minute period in control individuals and 0 6 ± 0 3 gm during the 75-minute period in the neoplastic group There was no significant difference between these means

Net rate of disappearance of glucose

The mean "total glucose" concentration ($\bar{G}_b$) was 180 ± 29 mgm per cent in the control individuals and 209 ± 40 mgm per cent in the neoplastic group In the control group the net rate of disappearance of "total glucose" from the blood ($G.D._b$) was 3.74 ± 1 04 mgm per 100 cc. per minute compared with a value of 2 34 ± 0 91 mgm per 100 cc. per minute for the individuals with neoplastic disease (Tables I and II) The difference between the mean $G.D._b$ and the difference

* Average values are given with one standard deviation.

† A significant difference between the means was taken to be a difference of at least 3.2 times the standard deviation of the difference between means ($p < 01$)

0.24 mgm per cent or 16 8 ± 5 9 per cent of the fasting value, and Lazarus, Volk, Jacobi, and Gilady (32) reported a mean maximum fall in normals of 14.2 per cent (10 to 22 5 per cent) These changes are more comparable to the findings in the patient group than in the normal individuals of the present study It should be noted that in previous reports as well as in the present data, the range of values for maximum fall in serum inorganic phosphate is large

The serum potassium concentration had a maximum fall of 0 41 ± 0 28 mEq per L or 10 8 ± 5 9 per cent of the fasting level in the control individuals In the patient group, the maximum decrease in serum potassium was 0 59 ± 0 32 mEq per L or 13 5 ± 7 5 per cent of the fasting concentration There was no statistically significant difference between the mean values for maximum fall, expressed in absolute or relative terms, in serum potassium concentration

DISCUSSION

A decrease in glucose tolerance, as indicated by a slower fractional rate of disappearance of blood glucose, was found to be associated with the presence of malignant disease The fasting blood sugar was within the normal range in these subjects The group of patients studied included individuals with lymphoma, chronic leukemia, and clinically localized carcinoma of the cervix, and carcinoma of the breast These individuals were carefully selected to exclude various other factors known to impair carbohydrate metabolism

The slower fractional rate of disappearance of glucose in the patients with malignant disease was associated with a higher mean blood sugar concentration following the intravenous administration of glucose The question arises, in view of these findings, as to whether the net amount of glucose disappearing from the blood in the neoplastic group differed from the control subjects The mean value for the net rate of disappearance of glucose was significantly lower in the patients compared with the control group However, in the patient group, a normal net rate of disappearance of glucose was more frequently observed than a normal fractional rate of disappearance of glucose There was no difference in the estimated volume of distribution of glucose between the patients and the control individuals These calculated values derived from the disappearance curve of glucose indicate that in certain neoplastic subjects, despite a decreased fractional rate of disappearance of glucose, the persistence of blood glucose concentrations at significantly higher levels than in normal subjects may be associated with an approximately normal net rate of disappearance of glucose A possible explanation of these findings is that net hepatic glucose output during the hyperglycemic phase is greater in the patients than in the control individuals and contributes to maintaining the higher blood glucose concentration This explanation does not exclude a concomitant decrease in tissue glucose utilization in these patients

The present data do not permit conclusions as to the mechanism of the decrease in glucose tolerance A slower fractional rate of disappearance of glucose, whether or not associated with a decreased net rate of disappearance of glucose, might result from an alteration in hepatic glucose metabolism, a decrease in peripheral utilization, or a disturbance in both of these metabolic processes

Peripheral glucose utilization has been found to be associated with a fall in serum inorganic phosphate following glucose administration (33, 34) A decrease in serum potassium concentration has also been reported to parallel glucose utilization (35) No statistically significant difference was observed between the control and patient groups with reference to the maximum fall *in the absolute* concentration of serum inorganic phosphate or serum potassium The mean fasting serum inorganic phosphate concentration in the patients with malignant disease was higher than in the control subjects The maximum fall in serum inorganic phosphate expressed as per cent of the fasting level was smaller in the patient group than in the control individuals It should be noted that in the patients with malignant disease, the maximum decrease in serum inorganic phosphate concentration and in serum potassium concentration was found to be variable, and in any given individual the magnitude of fall of these two ions did not parallel each other The meaning of these findings in terms of glucose metabolism is not apparent

The fact that the decrease in glucose tolerance

was associated with localized carcinoma, small in size relative to total body mass would make it unlikely that, in these patients at least, the decreased rate of disappearance of glucose was attributable to the carbohydrate metabolism of the tumor Indeed, Cori and Cori (36) demonstrated that tumor *in vivo* had an increased rate of glycolysis It would appear that the defect in glucose metabolism reflects alterations in host tissue metabolism associated with the presence of the neoplastic process In experimental animals there are several reports of defects in enzyme activity (37) including enzymes involved in carbohydrate metabolism (38), of tissues of the tumor bearing host

There are many studies on the incidence of cancer in diabetes mellitus (39) In general, these reports found that cancer occurs more frequently than expected in diabetics. The pertinence of this finding, if real, to the present data cannot be evaluated on the basis of available knowledge. The possibility does exist that the decrease in glucose tolerance in patients with malignant disease may reflect latent diabetes mellitus

It should be emphasized that the defect in carbohydrate metabolism as measured by the intravenous glucose tolerance test is not at all specific A decrease in glucose tolerance has been observed in a wide variety of conditions in addition to diabetes mellitus, such as endocrine disorders hepatic disease malnutrition infectious diseases neuropsychiatric disorders renal disease and obesity None of these conditions existed in the present group of patients with neoplastic disease Thus, this defect in glucose metabolism can be considered of no diagnostic value however its definition may shed some light on the metabolic alterations associated with neoplasia, as well as other conditions in which a decreased glucose tolerance may be present.

SUMMARY

1 Intravenous glucose tolerance tests were performed in 36 carefully selected patients with chronic leukemia lymphoma and clinically early epithelial neoplasms and in 19 subjects without cancer

2 In analyzing the glucose tolerance curves plotting either the log of the total blood sugar concentration against time, or the log of the blood sugar concentration in excess of the fasting value against time, provided an equally good index of glucose tolerance.

3 The patients with malignant disease compared with the control subjects had a significantly decreased fractional rate of disappearance of glucose and a significantly lower net rate of disappearance of glucose.

4 The fasting blood sugar concentration of the control and neoplastic groups did not differ significantly

5 No significant difference in the estimated volume distribution of glucose was found between the two groups

6 In the patients with malignant disease the fasting serum inorganic phosphate concentration was significantly greater and the maximum per cent fall in inorganic phosphate was significantly less than in the control individuals No significant difference was observed in the fall in serum potassium concentration between the two groups

ACKNOWLEDGMENT

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ABSORPTION OF WATER AND SODIUM FROM THE SMALL INTESTINE OF PATIENTS WITH NONTROPICAL SPRUE

By JOHN A. HIGGINS PHILIP R. LEE, JOHN F SCHOLER, RICHARD J REITEMEIER, CHARLES F CODE AND ERIC E. WOLLAEGER

(From the Sections of Medicine and Physiology Mayo Clinic and Mayo Foundation Rochester Minn)

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There is evidence that the absorption of water from the gastrointestinal tract of patients with nontropical sprue is delayed when they have recently eaten food (1) and also when they have fasted (2, 3) Excessive fecal loss of sodium has been demonstrated in patients with nontropical sprue (4), and recently evidence obtained by the use of isotopic sodium has indicated that the absorption of sodium chloride from the small bowel of patients with this disease is delayed (5)

The present study was undertaken to confirm if possible, the presence of a defect in the absorption of water or sodium in fasting patients with nontropical sprue and to obtain a more exact measurement of its degree. The study was made possible by the recent development of a method that allows the precise quantitative determination of the rate of absorption of isotopically labeled substances from the gastrointestinal tract of human beings under completely physiologic conditions (6)

METHODS

Fourteen patients who presented typical clinical, laboratory and roentgenographic features of nontropical sprue were studied. Three were men and 11 were women. Their ages ranged from 23 to 63 years with the majority being in the mid thirties. Rates of absorption were determined in most of the patients on only one occasion, the majority of these being obtained during relapses of the disease. Six tests were made during remissions.

A designation of the clinical status of the disease in each patient at the time the tests were performed has been used. When loss of weight, abdominal discomfort and diarrhea or any combination of such features of the disease predominated, the patient's clinical status was termed a "relapse." When such features were absent, or nearly so the term "remission" has been used. These terms are intended to provide only a very rough estimate of the severity of the disease in each of the patients at the time the test of absorption was made.

All observations were made in the morning The patients had eaten nothing since the meal of the previous evening In each test the isotopes were introduced into the upper part of the small bowel through a Sawyer tube after the position of the tube in the third portion of the duodenum had first been established by fluoroscopic examination. As a routine, about 10 gm. of barium sulfate was mixed with the test material and its distribution throughout the abdomen followed by frequent fluoroscopic and roentgenographic observation.

The tests made in the course of this study fall into two groups. In the first, the rate of absorption of water alone was measured eight such observations were made on five patients. In the second group the rates of absorption of both water and sodium were determined. In addition to the dual determinations, refinements in methodology developed during the progress of the second part of the study allowed more precise determination of the rates of absorption of both water and sodium.

The method (6) employed for estimation of the rate of absorption of a labeled substance requires determination of its rate of appearance in the arterial blood while it is being absorbed as well as its rate of disappearance from the arterial blood stream after its rapid intravenous injection. The precise rate of absorption of the isotope is then calculated by integration of these two rates In the past it has been necessary to employ mean rates of arterial disappearance of the isotopes as determined in a group of healthy persons (6–8) and this was the procedure adopted in the first group of observations presented in this report. While use of a mean rate of arterial disappearance has been appropriate for the study of normal human beings since rates of arterial disappearance vary so little among healthy persons it was recognized at the outset that it might not be as applicable to the study of patients who are sick. The first step toward correction of this weakness was to determine the rate of disappearance of each isotope following its intravenous injection a few days before, or after the determination of its rate of appearance in the arterial blood during absorption from the bowel. This procedure was adopted in four of the nine patients of the second series a further improvement was made in the tests on the five remaining patients. In these, dual isotopes of water (deuterium and tritium oxide) and dual isotopes of radiosodium (sodium[22] and sodium[24]) were employed (9) One of the isotopes of each pair was given intravenously while

simultaneously the other of each pair was placed in the small bowel Determination of the concentration of the isotopes in the same samples of arterial blood then yielded simultaneous appearance and disappearance rates for both water and sodium. These rates were then employed in the calculation of the rate of absorption of each of the isotopes The procedures followed in carrying out these tests did not differ significantly from those followed when single isotopes were used (6) The methods followed in analyzing for the dual isotopes in the blood and the validity of the procedures were first established by experiments on dogs and then applied to tests on human beings (9)

The quantities of the isotopes used in the test ranged from 19 to 50 gm of deuterium oxide (D_2O), 2 5 to 3 0 millicuries of tritium oxide, 10 to 25 microcuries of radiosodium[22] and 14 to 100 microcuries of radiosodium[24] The radiosodium was routinely dissolved in the labeled water and sufficient sodium chloride was added to make the solution isotonic. Zero time in the test was taken as the midpoint of the injection of the isotopes, those into the bowel being synchronized with those into the vein Samples of arterial blood were drawn at minute intervals for 12 minutes and then less frequently until the termination of the test at 1 to 2 hours Samples of blood for determination of the 3-hour or 24-hour equilibrium values were drawn by separate venipuncture. The concentrations of the isotopes in the blood were determined according to methods that have been described previously [deuterium with a mass spectrometer (10, 11), tritium with a liquid scintillation counter (12) and radiosodium in a well-type sodium iodide (thallium) scintillation counter]

Calculation of the rates of absorption of the isotopes was carried out according to the procedure previously described (6) except that in the last group of tests the time intervals used in the calculations were changed. The concentrations of the isotopes at 1½ 3½, 4½ 5½ minutes and so on, up to 11½ minutes, and the concentrations at 14 minutes and every 4 minutes thereafter up to 50 and then every 10 minutes for the remainder of the first 1½ hours were employed instead of those previously reported. This represents a slightly different time scheduling from that employed previously in the calculation. The change has been found to improve the determination of the rate of absorption of sodium Such rather minor factors have been brought into focus by elimination of the use of a mean disappearance curve and adoption of the more accurate procedure of using the disappearance curve belonging to each individual, particularly when this is determined at the same time as the test of absorption. The concentration of the labeled water in the venous blood 3 hours after its administration was used as the equilibrium value in the calculations In the case of sodium, the concentration in the venous blood at 24 hours was used as the equilibrium value in the majority of the tests, use of a 3-hour to 9 hour equilibrium in a few instances did not significantly alter the results In each determination the percentages of the isotopes absorbed as the test progressed were plotted, and the slope of the straight line that best fitted the points which included the absorption of at least the first 50 per cent of the isotopes was expressed as the initial rate of absorption, in addition, the time required for the absorption of the first 50 per cent and 67 per cent of the administered amount of each of the isotopes was recorded.

RESULTS

Patients in whom absorption of water alone was tested

In this group of tests a mean rate of disappearance of deuterium oxide from arterial blood as determined in a series of normal persons was used in the calculation of the rate of absorption The results may be compared directly with those obtained previously in our laboratory with the same technic (6–8) We have calculated the mean values for the total of 29 healthy persons tested in

TABLE I

Group 1—Rate of absorption of water from small bowel of patients with sprue

Case	Test	Initial rate % absorbed per minute	Minutes required for absorption of 50%	67%	Clinical status
1	126	21 7	2 8	4 7	In remission
	113	13 5	4 4	6 4	In remission, relapsed 5 days later
2	119	7 1	6 8	12 8	In relapse
3	125	7 8*	6 4	9 6	In relapse
	112	10 0	5 3	9 1	Recovering from relapse
4	74	10 9	4 9	6 9	Recovering from relapse
5	147	14 7	4 3	6 4	In remission
Controls†		23±6	2 8±0 7	4 6±1 3	

* Hypertonic solution placed in bowel

† Series of 29 persons from studies by Lee, Code and Scholer (7) and by Reitemeier, Code, and Orvis (8) values following the ± sign are the standard deviations of the means

TABLE II

Group 2—Rate of absorption of water from small bowel of patients with sprue

Case	Test	Initial rate, % absorbed per minute	Minutes required for absorption of 50%	Minutes required for absorption of 67%	Clinical status
6	234	6 4	7.5	13 1	Relapse
7	271	7 1	6 9	10.8	Relapse
9	274	9 4	5 5	9.2	Relapse
10	259	10 4	5.3	10 4	Remission
11	198	20 0	3 1	4.0	Recovering from relapse
12	236	21 7	2 3	3 1	Recovering from relapse
13	254	25 0	2.7	3 4	Remission
14	262	26 3	1 9	2 6	Remission

these earlier studies, the mean initial rate of water absorption, per minute was 23 per cent of the administered water (standard error of mean 1 1) the slowest rate was 14 8 per cent per minute. All of the patients with sprue who were in relapse had rates that were slower than this (Table I) In general, the rate of water absorption paralleled the clinical condition of the patient the slowest rates of absorption being encountered in patients in relapse and the most rapid rates in those recovering or in remission This generalization was also reflected in the time required for absorption of 50 per cent and 67 per cent of the administered water

Patients in whom rates of absorption of both water and sodium were tested simultaneously

In this group of tests the rates of disappearance of water and sodium from arterial blood were determined individually for each patient thereby eliminating the use of a mean disappearance rate in the calculation of the rate of absorption The initial rates of absorption of water in three healthy persons using this refinement in technic were 16 21 and 24 per cent per minute which values are within the ranges of those obtained in normal persons with the less accurate procedure Once again, in this more accurate series of observations all of the patients in relapse had rates of absorption of water that were slower than those of normal persons and there was in general a parallelism between the rate of absorption of water from the small bowel and the condition of the patient (Table II) The combined results of the two groups of observations allow the general conclusion that the rate of absorption of water from the small bowel of patients with sprue while in relapse and under fasting conditions is slower than that of healthy persons but when the patients are in a remission the rate approaches or becomes equal to that of normal persons

The rate of absorption of sodium from the small bowel of the patients with sprue was even more decisively retarded than was the absorption of water Tests of the rate of absorption of sodium have been made on 12 healthy persons in the course of other studies (unpublished data) The mean initial rate of absorption from the small bowel of the group was 9 5 per cent per minute and the slowest rate encountered was 6 per cent per minute. Only one of the patients with sprue had a rate of absorption that fell within the normal range and this patient had a very mild form of the disease and was in remission at the time of the test (case 14 Table III) All of the patients studied during a relapse had exceedingly slow rates of absorption ranging from 0 6 to 3 8 per cent per minute and once again there was a rough correlation between the degree of impairment of the absorption of sodium and the clinical condition of the patient

Hypomotility

The 10 gm of barium sulfate suspended in the labeled water and placed in the small bowel did not allow a very precise estimate of motility of the small bowel but it was sufficient to demonstrate the decisive roentgenographic difference shown previously by others between sprue patients in relapse and healthy persons In the patients the barium spread very slowly through the abdomen and often remained 'puddled in the upper part

TABLE II

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7	271	7 1	6.9	10 8	Relapse
9	274	9 4	5 5	9.2	Relapse
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TABLE III

Group 2—Rate of absorption of sodium from small bowel of patients with sprue

Case	Test	Initial rate % absorbed per minute	Minutes required for absorption of 50%	Minutes required for absorption of 67%	Clinical status
6	234	0 6	80 0	112 5	Relapse
7	271	0 7	>85 0		Relapse
8	188	3 8	21 8	49 7	Relapse
9	274	0 8	>85 0		Relapse
10	259	0 7	97 0	115 0	Remission
11	198	4 0	15 4	35 5	Recovering from relapse
12	236	2 8	18 7	29 2	Recovering from relapse
13	254	5 7	10 1	14 0	Remission
14	262	10 4	4 6	6 5	Remission
Mean of 12 healthy persons*		9 5	6 3	8 0	

* Series from study by Reitemeier, Code, and Orvis (8)

of the small bowel throughout most of the test, whereas in healthy persons the same amount of barium suspension was quickly distributed over a wide area

DISCUSSION

It should be emphasized at the outset that intestinal absorption as determined by the technics employed in this study is the unidirectional passage of the labeled material from the lumen of the bowel to the arterial blood It is not a measure of the net result of exchanges in both directions across the intestinal mucosa which has often been used by others as the index of absorption

Wollaeger and Scribner (1) found that absorption of water was retarded in patients with sprue when the water was taken during or following a meal The results obtained in the present study demonstrate that the absorption of water is retarded in patients with sprue even in the absence of food These results confirm the earlier findings of Taylor (3) They indicate in addition that the degree of retardation parallels in a rough way the condition of the patient, being greatest during exacerbations of the disease The present study also confirms the findings of Newsholme and French (5) that there is a delay in the absorption of sodium from the small intestine of patients with sprue The degree of the defect of absorption is greater for sodium than for water The question arises, "Is sodium the anchor that holds the water in the bowel?" It seems likely that other factors are also involved

Although this investigation demonstrates slowed absorption of water and sodium from the small bowel of patients with nontropical sprue, it does not define the mechanism of the defect Reduced motility was certainly present in the small bowel of all of our patients in relapse It has been shown by others that the absorption of glucose, methionine (13) and vitamin A (14) from the small bowel of human beings is slowed or hastened by decreases or increases in the motor action of the small intestine Higgins, Code, and Orvis (15) have recently found that the absorption of water and sodium from the upper part of the small bowel *is retarded in healthy persons when propulsive* motility is reduced or eliminated by the administration of methantheline bromide It seems likely that the reduced motility of the small bowel of the patients we studied contributed to the slowed absorption of both water and sodium Other factors, however, were most likely also involved, for the much greater retardation of the absorption of sodium than of water suggests additional and more specific defects such as might occur in the membrane or membranes separating blood from bowel contents

SUMMARY

The rate of absorption of isotopically labeled water and sodium from the small bowel in 14 patients with nontropical sprue was abnormally slow when the patients were tested during relapse of their disease When the patients were tested during remission, the rates approached or became equal to those of healthy persons

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ADAPTIVE VALUE OF RESPIRATORY ADJUSTMENTS TO SHUNT HYPOXIA AND TO ALTITUDE HYPOXIA [1]

By GEORGE HUSSON [2] AND A B OTIS [3]

(*From the Department of Surgery, The Johns Hopkins University School of Medicine, Baltimore, Md*)

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Only a few adaptive mechanisms by which the body responds to hypoxia are at present understood well enough to be quantitatively examined and evaluated Among these are the alterations that occur in the pulmonary ventilation, the acid base balance of the blood and the oxygen carrying capacity of the blood It is the purpose of this paper to compare the adaptive value of these adjustments, as judged by their effectiveness in raising the partial pressure of oxygen in blood and tissues, in two types of hypoxia 1) that arising from chronic exposures to a low P_{O_2} in the environment (altitude hypoxia) and 2) that arising from the presence of a right to left shunt in the circulation (shunt hypoxia) We shall attempt to show that although the changes which occur in these two types of hypoxia are qualitatively similar in some respects, there are important quantitative differences in their adaptive value We shall also present evidence that in the case of shunt hypoxia there is no reduction in the basal oxygen requirement of the body For previous work by other authors pertinent to our general subject see references 1 through 4 More detailed data and a description of methods used in this investigation may be found in references 5 through 8

Pulmonary ventilation

Numerous investigators have shown that individuals who are chronically hypoxic from prolonged residence at high altitudes have an increased ventilation which is greater the higher the altitude There is evidence in the literature that individuals who are hypoxic because of circulatory shunts also ventilate more than normal

We have measured the total resting ventilation of individuals with shunt hypoxia and have confirmed the presence of hyperventilation (6) In a smaller number we have made measurements of the resting alveolar ventilation The results are shown as points in Figure 1 For comparison a curve previously published by Rahn and Otis (9) and indicating the magnitude of alveolar ventilation as a function of alveolar P_{O_2} in healthy individuals who had lived at altitude for relatively extended periods is also shown Although our data show a rather large variation among individuals

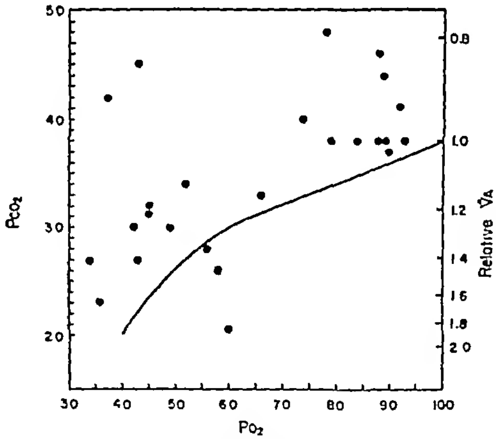

FIG 1 ALVEOLAR P_{CO_2} AND RELATIVE ALVEOLAR VENTILATION AS A FUNCTION OF ARTERIAL P_{O_2}

The solid circles represent measurements made on patients with congenital heart disease. Those with a P_{O_2} less than 75 mm had right to left circulatory shunts, those with a P_{O_2} greater than 75 mm did not. The alveolar P_{CO_2} was either directly determined from analysis of end tidal samples or calculated from expired P_{CO_2} and estimated dead space. The curve is taken from Rahn and Otis (9) and applies to normal individuals acclimatized to various altitudes Relative alveolar ventilation was calculated as follows

$$\dot{V}_A = \frac{38 \text{ mm}}{P_A{}'_{CO_2}}$$

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[2] Present address Department of Pediatrics, Syracuse Memorial Hospital, Syracuse 10, New York.

[3] Present address College of Medicine, University of Florida, Gainesville, Florida.

they demonstrate a definite tendency for the alveolar ventilation to increase with decreasing arterial P_{O_2}, the relationship being similar in form but less in extent to that found for the altitude dwellers

The hyperpnea of the altitude dweller constitutes for him an important adaptation. If at 15,000 feet for example, an individual did not increase his ventilation above the sea level value, his alveolar (and arterial) P_{O_2} would be about 38 mm Hg His arterial oxygen saturation would be about 72 per cent. The increase in ventilation that usually occurs in the individual acclimatized to this altitude is such as to increase his arterial P_{O_2} to about 50 mm Hg and his arterial saturation to about 83 per cent an increase which is really significant as an adaptive mechanism

The victim of shunt hypoxia on the other hand, can increase the oxygenation of his arterial blood but little by hyperpnea. His pulmonary venous blood will be about 97 per cent saturated even with a normal ventilation, and although increasing the ventilation will raise the P_{O_2} of this blood it will alter the per cent saturation only insignificantly be-

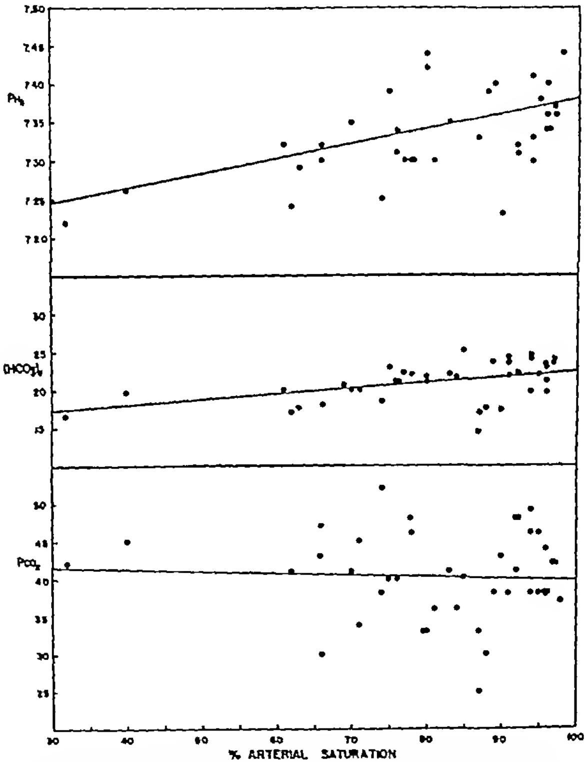

FIG. 2. PLASMA pH PLASMA BICARBONATE CONCENTRATION IN MILLIMOLS PER LITER AND PARTIAL PRESSURE OF CARBON DIOXIDE IN ARTERIAL BLOOD AS A FUNCTION OF ARTERIAL OXYGEN SATURATION

cause of the flatness of the dissociation curve in this region. Furthermore that component of this mixed arterial blood which is shunted around his lungs will not be directly affected at all. Resting hyperpnea in such an individual would therefore seem to be of negligible adaptive value as far as improvement of oxygenation of his systemic arterial blood is concerned.

However, the presence of a right to left shunt introduces a problem in CO_2 elimination as well as of oxygen uptake. With a normal resting ventilation the pulmonary venous P_{CO_2} will be normal but the arterial P_{CO_2} will be higher and arterial pH lower than normal because of the admixture of venous blood. A normal arterial P_{CO_2} and pH can be maintained only by hyperventilation of the proper magnitude. With no increase in ventilation a normal pH, but elevated P_{CO_2}, could be maintained by an increase in the alkaline reserve. It is therefore of interest to know exactly how the acid base balance is adjusted in shunt hypoxia.

Acid base balance

We have measured arterial pH, and plasma bicarbonate and have calculated the arterial P_{CO_2} in 35 individuals with shunt hypoxia and in 12 nonhypoxic individuals. The results are shown in Figure 2 in which each of the above variables is plotted as a function of the arterial O_2 saturation. The following regression lines have been calculated.

$$\text{pH} = 0.00189\ (\%\ \text{Sat.}) + 7.19$$
Std. error of estimate = 0.05

$$(\text{HCO}_3) = 0.074\ (\%\ \text{Sat.}) + 15.09$$
Std. error of estimate = 2.54

$$P_{CO_2} = 42.23 - 0.0234\ (\%\ \text{Sat.})$$
Std. error of estimate = 5.4

The interrelationship among these variables is graphically presented in Figure 3 on the pH-bicarbonate diagram of Davenport (10). The data are widely scattered and values from the nonhypoxic and hypoxic groups overlap to some extent but the points representing the hypoxic group tend to fall in the region of the chart below the normal pH and below the normal buffer line, and so indicate metabolic acidosis.

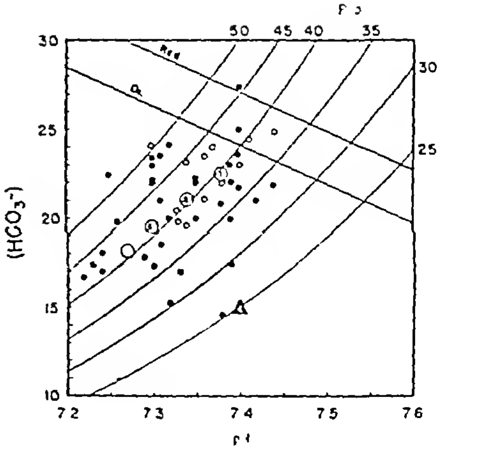

FIG. 3. RELATIONSHIP BETWEEN PLASMA BICARBONATE (MILLIMOLS PER LITER), PH AND P_{CO_2} MM. HG

Closed circles represent subjects with and open circles subjects without shunt hypoxia. The lines labelled red. and ox. are standard dissociation curves from Davenport (10).

The large circles on the chart labelled 1.0, 0.8, 0.6, 0.4 are calculated from the regression equations for 100 per cent, 80 per cent, 60 per cent, and 40 per cent saturation, respectively, and their position indicates a tendency for metabolic acidosis to increase progressively with hypoxia. The position of the triangle represents the acid-base picture of residents at high altitude who have a per cent saturation of about 75 (11). There is no doubt that the average congenital cyanotic individual in our group has a different acid-base adjustment than the altitude dweller with a similar degree of hypoxia.

Figure 2 indicates that the alkali reserve of individuals with shunt hypoxia tends to be reduced in proportion to the degree of hypoxia but to a less extent and for a different reason than is that of altitude dwellers who are chronically hypoxic to a similar degree. Our subjects tend to maintain an alveolar P_{CO_2} of about 40 mm. on the average, whereas the hypoxic altitude dweller has a much lower P_{CO_2}. Consequently, the average congenital cyanotic individual appears to be in a state of metabolic acidosis which is only partially compensated by an increased ventilation. Such a tendency toward metabolic acidosis in congenitally cyanotic individuals has been reported previously by Morse and Cassells (12).

The acid base picture in chronic altitude hypoxia is by contrast, one of compensated respiratory alkalosis This situation enables the mountain dweller to load his arterial blood at a relatively high P_{O_2} and at the same time to maintain a normal pH.

Although the mechanisms and the sequence of events involved in the acid base adjustments to altitude hypoxia seem reasonably well understood, the same cannot be said for the individual who is hypoxic from congenital heart disease. It is not clear, for example, why the respiratory center does not respond sufficiently to relieve the acidosis that tends to be present in these individuals Regardless of its origin it may be that an acidosis is of advantage to the congenital cyanotic because it would tend to aid in the unloading of oxygen in the tissues without hindering to a comparable degree the loading of oxygen in the lungs Reference to the nomograms of Dill, Talbott Consolazio and Edwards (11 13) will make this clear

Consider an individual whose arterial saturation is 65 per cent because of the presence of a right to left shunt The blood leaving his lungs will be almost completely saturated (say 97 per cent) whatever the pH, since the Bohr effect is very small at high saturations If the pH of the mixed arterial blood beyond the shunt were 7 4 the P_{O_2} at 65 per cent saturation would be 34 mm On the other hand if the pH were 7.25 the P_{O_2} at the same saturation would be 39 mm The P_{O_2} of mixed venous blood leaving the tissue would be affected to about the same degree, and we may conclude that the presence of the acidosis resulted in delivery of O_2 to the tissues at a pressure about 5 mm. higher than would have occurred at a normal pH

Thus it appears that the acidosis observed in some congenital cyanotic individuals may be considered as an advantageous adaptation insofar as delivery of O_2 to the tissues is concerned although it may of course be disadvantageous in other regards

One might ask whether acidosis would similarly be of benefit in altitude hypoxia The answer seems to be that it would not, because in the hypoxia of high altitude the blood leaving the lungs is at a relatively low saturation and the Bohr effect would hinder the loading of O_2 in the lungs sufficiently to offset any advantage to unloading in the tissues

This can be illustrated by a specific example again with reference to Dill, Talbott and Consolazio s (11) nomogram. Consider an individual at altitude, who has an oxygen carrying capacity of 30 volumes per cent, who is ventilating at such a rate that his arterial P_{O_2} is 33 mm., and whose A-V oxygen difference is 5 volumes per cent. At pH 7 4 the arterial saturation will be 65 per cent and the mixed venous saturation 48 per cent corresponding to P_{O_2} s of 33 mm. and 25 mm. respectively At pH 7.25 the arterial saturation will be 56 per cent and the mixed venous saturation 40 per cent corresponding to P_{O_2}'s of 33 and 25 mm., respectively Thus an acidosis is evidently of no advantage to the delivery of O_2 in the case of altitude hypoxia.

The increase in oxygen carrying capacity with chronic hypoxia

It is well known that individuals who are chronically hypoxic tend to develop a polycythemia with a concomitant increase in the oxygen carrying capacity of the blood. The mechanism by which the increased hemoglobin is brought about is still obscure nor is there general agreement as to its importance in the overall picture of acclimatization (14)

Data showing the magnitude of the increase in oxygen carrying capacity of the blood in man residing at various altitudes have been summarized by Hurtado (15) In Figure 4 we have plotted these data to show the oxygen carrying capacity as a function of the per cent saturation of the arterial blood. It is evident that an excellent linear relationship exists the equation for the straight line being

$$C\ max = 67\ 1 - 0\ 476\ Sa \qquad (1)$$

where C max is the oxygen carrying capacity in volumes per cent and Sa is the per cent saturation of arterial blood

The combined O_2 carried by the arterial blood is the product of capacity times saturation or in this case

$$CaO_2 = 67\ 1\ Sa - 0\ 476\ Sa^2 \qquad (2)$$

Values for combined oxygen have also been plotted on Figure 4 as well as a curve calculated

In contrast to these striking results normal or slightly elevated values for basal oxygen consumption in congenital cyanotic heart disease have been obtained by Holling and Zak (17), Burchell, Taylor, Knutson, and Wood (18), Ernsting and Shephard (2) and Davison, Armitage, and Arnott (4). Thus the majority of the evidence indicates that no diminution in basal oxygen requirement occurs in chronic hypoxia resulting from congenital cyanotic heart disease, but the question is of such fundamental importance that we decided to satisfy our curiosity by measurements of our own.

None of the above mentioned investigators made measurements of oxygen consumption on a control group of acyanotic individuals but used "standard values" for comparison. Consequently, for our own investigation we decided to include a suitable control series.

Our chronically hypoxic series consisted of 42 individuals with cyanotic congenital heart disease. The arterial oxygen saturation in this group ranged from 61 per cent to 93 per cent and averaged 83 per cent. Our control series was composed of 45 individuals with non-cyanotic congenital heart disease, and arterial oxygen saturations of 94 per cent or higher. Various age groups were represented in both series. The measurements of oxygen consumption were made in connection with the procedure of cardiac catheterization which was being performed for diagnostic purposes. The subjects, who were in a fasting state, had received shortly before the procedure premedication consisting of morphine sulfate, 1 mg per 5 Kg body weight and scopalamine, 0.05 to 0.1 mg per 5 Kg body weight, and in the age groups up to 15 years Nembutal® suppositories, 2 mg per Kg body weight. The patients over 15 years usually received Nembutal® orally in a dose of 0.1 gm. Oxygen consumption measurements were based on the analysis of expired gas collected in a 100-liter recording spirometer. After two or three preliminary wash-out periods, the final collection was made during a 3-minute period. Analysis of the expired gas was performed on the Scholander (19) apparatus. Body surface area was estimated from height and weight according to Dubois (20). Oxygen saturation was determined by a modification of the Nahas (21) technique on blood samples drawn by arterial puncture.

RESULTS

Since it is well known that the resting oxygen consumption per square meter of surface area varies considerably with age, allowance for this was made in the interpretation of our data. The subjects in each series (acyanotic and cyanotic) were divided into five age groups and the mean oxygen consumption was determined for each group. The results are represented graphically in Figure 7. Data from Robinson (22) for the resting oxygen consumption of normal males of comparable ages are also shown for comparison.

It is clear that up to age 20 our data for both the cyanotic and acyanotic series agree remarkably well with those of Robinson. In the higher age groups, however, our values, while still showing no significant difference between cyanotic and acyanotic patients, deviate significantly from those of Robinson's. We are not certain as to the explanation for this discrepancy, but offer the suggestion that the older age groups were not as completely relaxed during the procedure partly perhaps because of less effective sedation and partly because of greater apprehension arising from more awareness of the potential hazards of cardiac catheterization.

At any rate, the important fact from the point of view of the present investigation is that there is no significant difference between the oxygen con-

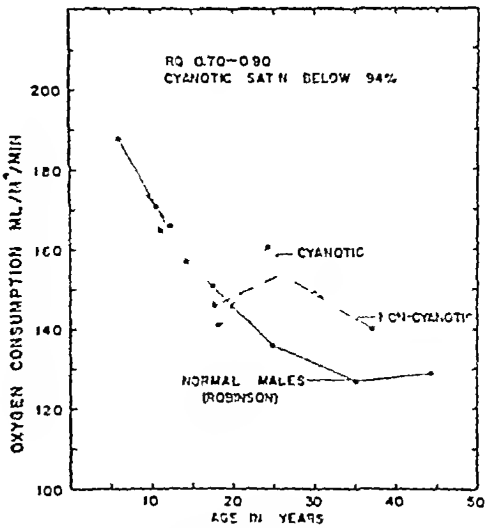

FIG 7. RELATIONSHIP BETWEEN RESTING OXYGEN CONSUMPTION AND AGE IN CYANOTIC AND NON-CYANOTIC SUBJECTS

sumption of patients with chronic hypoxia arising from circulatory anomalies and that of those who had circulatory anomalies but no hypoxia. This is true whether the comparison is made between the two series as a whole or between similar age groups from the two series

We have also compared our two series in another fashion on the basis of basal metabolic rates estimated by application of the Dubois (20) nomogram to our measured values for oxygen consumption. This comparison shows that, although in each series there is a slightly higher than normal B.M.R. value, the difference between the two series is not significant.

From this study we can only infer that the resting oxygen requirement is not depressed in our chronically hypoxic subjects and so are in agreement with the majority of other investigators mentioned in the introduction, and at variance with the findings of Bing Vandam Handelsman, Campbell, Spencer and Griswold (1) Whether the low values for oxygen consumption obtained by Bing mean that his hypoxic subjects were vastly different from those studied by others or whether they were due to some systematic difference in his technique of measurement is a question that cannot be answered Only one of our subjects had a B M.R as low as the average of Bing s group Possibly Bing s subjects were more heavily sedated during the test, but no mention of sedation is made in his published reports The objection might be raised that our cyanotic subjects were, on the average less hypoxic than were Bing's and that one therefore might expect less of a depression of metabolism in our group This argument appears weak, however in view of the fact that his data show no significant correlation between degree of hypoxia (as estimated by arterial saturation) and depression of the basal metabolic rate It is unfortunate that Bing s study did not include measurements on a control group of noncyanotic subjects

COMMENT

We have examined only a few of the many bodily adjustments that may occur as a result of exposure to chronic hypoxia and have evaluated these on the basis of a single criterion their effectiveness in elevating the P_{O_2} of the body Other adjustments may be of equal or even greater importance and other criteria for their evaluation may prove more useful (23)

We have deliberately omitted any consideration of cardiac output because in the case of shunt hypoxia, there is not sufficient information available as to how an alteration in cardiac output is distributed between systemic and pulmonary flows when a shunt is present (4 18)

Adaptations are by their nature, compromises in that concessions must be made in return for the advantages gained. For example, there is evidence that polycythemia is a predisposing factor in the formation of pulmonary and cerebral thrombi (24 25) The importance and probability of occurrence of such undesirable effects must of course be taken into account in any complete evaluation of an adaptive mechanism, especially when individual cases are concerned

SUMMARY

Although an increased pulmonary ventilation is an important adaptation to altitude hypoxia it is of negligible value in raising the P_{O_2} of the body in shunt hypoxia. It may be of importance in the optimal regulation of acid base balance in the latter instance however In chronic altitude hypoxia the usual acid base balance is one of compensated respiratory alkalosis In shunt hypoxia there is a tendency for metabolic acidosis to be present. This may be of advantage in aiding the unloading of oxygen from blood to tissues Polycythemia, which is usually present in both altitude and shunt hypoxia is a more effective adaptation in the latter type because here it can raise the arterial as well as the mixed venous P_{O_2} The basal oxygen requirement of individuals with shunt hypoxia does not appear to be lower than the normal.

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GASTROINTESTINAL WATER AND ELECTROLYTES III THE EQUILIBRATION OF RADIOBROMIDE IN GASTROINTESTINAL CONTENTS AND THE PROPORTION OF EXCHANGEABLE CHLORIDE (Cl_e) IN THE GASTROINTESTINAL TRACT[1]

By N J SWEET J NADELL[2] AND I S EDELMAN[3]

(From the Department of Medicine University of California School of Medicine and the San Francisco Hospital San Francisco Calif)

(Submitted for publication July 27 1956 accepted October 15 1956)

Although chloride is distributed chiefly in the extracellular fluid, it is also found in intracellular water in as yet poorly defined quantities The extracellular distribution includes plasma, free interstitial fluid the interstitial fluid of dense connective tissue and bone and transcellular fluid The latter has been proposed as that portion of extracellular fluid formed, at least in part as a result of active cellular transport mechanisms (1) The chemical composition of gastrointestinal contents differs in many ways from a simple ultrafiltrate of plasma. There is good evidence that chloride flux across the gastrointestinal mucosa either directly or indirectly involves active transport mechanisms (2, 3)

The physiologic significance of the chloride in transcellular fluid depends on its quantity and on the extent and rate of its equilibration with total exchangeable chloride, as well as other factors The large volume of chloride-containing fluid in the gastrointestinal tract (4, 5) is one reason for the quantitative and functional definition of this moiety in relation to total body chloride

Radiobromide (Br^{82}) was used as the tracer material in this study because of some disadvantageous physical characteristics of chloride^{36} and chloride^{38} The half life of Cl^{36} is 4×10^5 years (6) making it a potential hazard in terms of laboratory contamination and disposal Cl^{38} has a half life of only 37 minutes which limits its value in studies requiring an equilibration period of more than a few hours (6)

The bromide chloride ratio has been shown to be the same in tissues as in plasma after distribution equilibrium in animals, except for brain and cerebrospinal fluid (7–9) On the basis of these observations stable and isotopic bromide has been used extensively for *in vivo* estimations of total exchangeable chloride (Cl_e) (7, 10–15)

This communication presents observation on a) the fraction of total exchangeable chloride (Cl_e) in the lumen of the gastrointestinal tract of normal rabbits b) the exchangeability of this chloride fraction based on bromide partition and c) the amount of chloride in the gastrointestinal tract of man at post mortem examination

METHODS

A Rabbits

Thirty-eight adult albino rabbits were studied in pairs, consisting of a male and a non-gravid female. The animals were allowed *ad libitum* ingestion of water but food was withheld from the time of isotope injection until sacrifice. The fasting periods varied from 21 to 65 hours.

Each animal was injected intraperitoneally with 15 to 25 microcuries of KBr^{82} from calibrated syringes.[4] The injected material was made up as a neutral, sterile isotonic solution with saline. Observations on half lives of decay on aliquots of the injected material fell within the reported values for Br^{82} (6) indicating that any small quantities of K^{42} present were not contributing significantly to radioassay

[1] This work was carried out with the support of grants from the American Heart Association, the United States Public Health Service (No H 1441) the Fleischmann Foundation, the San Francisco Heart Association and the Paul and Susan Gardiner Fund.

[2] Research Fellow of the National Heart Institute, U S Public Health Service.

[3] Established Investigator of the American Heart Association.

[4] KBr^{82} was supplied by the Oak Ridge National Laboratory of the Atomic Energy Commission. On the day of shipment each unit contained 5 millicuries of K^{42} and 120 millicuries of Br^{82} Five half lives for K^{42} were allowed to elapse prior to use, insuring negligible contamination. Br^{82} is a β^- γ emitter with a half life of 35.7 hours (6)

The techniques of collection of blood, bladder urine, stool and gastrointestinal contents were described in previous communications (1, 16) Urine and stool passed during the period of isotope equilibration were collected quantitatively in metabolism cages. In eight instances erythrocytes were aspirated from below the plasma layer of centrifuged heparinized blood obtained by cardiac puncture. The erythrocytes were hemolyzed in four times their volume of distilled water, and the hemolysates were filtered through double thickness paper No attempt was made to remove trapped plasma, since the error due to plasma contamination in the specific activity of erythrocyte chloride would not exceed 10 per cent. Excreted stool and fecal contents of the distal portion of the large bowel were homogenized in a Waring blendor with measured volumes of distilled water, and filtrates collected after passage through glass-wool Aliquots of plasma, lysed erythrocytes, bladder urine, cage urine, excreted stool, stool in the descending colon and gastrointestinal contents were taken for assay of radioactivity and chemical analysis No attempt was made to prevent volatilization of HBr by neutralization of gastric contents during the brief exposure to room air

One-ml aliquots of all samples were plated on filter paper in metal planchets in triplicate, dried under an infrared lamp and covered with parafilm. Standards of three separate dilutions were prepared from aliquots of each of the injected solutions Triplicate one-ml. plates were made from each dilution as above. It was found that addition of one drop of concentrated detergent solution to the standards and one or two drops of 50 per cent sucrose solution to all planchets improved the reproducibility of radioassay

Assay of radioactivity was carried out with an end-window, thallium-activated sodium-iodide scintillation counter Coincidence and self-absorption losses were found to be negligible. Consequently, these corrections were not applied. Corrections for background radiation and decay were applied to each assay

Serum, urine, stool and gastrointestinal contents were analyzed for chloride content in triplicate by the Wilson and Ball method (17) Iced containers were used to sharpen the end-points

B Human subjects

Thirteen human subjects were studied at the time of post mortem examination The age, sex, weight and height of each subject was recorded. The criteria for patient selection and the post mortem pathologic findings were described in the first report in this series (1) Direct collections were achieved by emptying each segment of the gastrointestinal tract. The details of collection and processing of these samples have been reported previously (1) A thin layer of mucus invariably clung to the mucosal wall of each segment. Quantitative emptying of each segment was not attempted to avoid contamination of the samples with gastrointestinal epithelium or blood.

Triplicate chloride analyses were carried out on aliquots of the diluted contents collected from each segment of the gastrointestinal tract by the Wilson and Ball method (17)

CALCULATIONS

A Rabbits

Bromide is, for the most part, partitioned in direct proportion to chloride in body fluids (7–9) The application of radiobromide (Br^{82}) dilution to the measurement of total exchangeable body chloride content is justified on the same grounds as the use of bromide (Br$^{79/81}$) for this purpose, and yields results which closely approximate those obtained with radiochloride (Cl$^{36/38}$) (7, 14, 15)

The total exchangeable body chloride content (Cl$_e$) was calculated with the conventional dilution formula (1), corrected for urinary losses Cardiac blood samples were drawn 21 to 65 hours after injection of Br^{82} Urinary loss of Br^{82} was extremely variable in spite of the fact that the animals were matched for age, sex and body weight and kept on identical diets prior to fasting In 14 rabbits fasted for 21 to 27 hours the cumulative urinary Br^{82} excretion rate averaged 8 1 per cent of the injected amount per day, with a range of 1 0 to 15.2 per cent per day, in 17 rabbits fasted for 41 to 48 hours the cumulative urinary Br^{82} excretion rate averaged 9 4 per cent of the injected amount per day, with a range of 5 1 to 22.2 per cent per day, while the two rabbits subjected to 64- and 65-hour fasts excreted Br^{82} at a rate of 4 7 and 3 6 per cent of the injected amount per day, respectively

The partition of Br^{82} in proportion to chloride in gastrointestinal contents and erythrocytes was evaluated by relating the Br^{82} chloride concentration of these samples to the Br^{82} chloride concentration in serum and expressed as the specific activity ratio (S.A R.) The S.A.R. is indicative of the fractional exchange of the bromide tracer relative to chloride, and exchange equilibrium is assumed to be complete when a value of 1 00 is obtained consistently (1, 18)

B Human subjects

The intraluminal gastrointestinal chloride content has been expressed as an absolute quantity (mEq), as the amount per unit of body weight (mEq per kgm.) and as a percentage of the predicted total exchangeable chloride content (per cent of Cl$_e$) (cf Table IX) The total exchangeable chloride content was predicted from previously published normal values (11, 12, 19) The available data on Cl$_e$ in normal human subjects are too few in number to permit predictions corrected for age or body habitus We have, therefore, applied single standards, taking into account only sex and body weight. It was assumed that the Cl$_e$ was 29 0 mEq per kgm and 30 9 mEq per kgm of body weight in female and male subjects, respectively There is abundant evidence from chloride balance data in humans to indicate that these predictions probably do not involve errors in excess of a factor of two The need to establish some estimate of gastrointestinal chloride pool size in terms of total amount of chloride in the body in humans justifies these crude approximations

TABLE I

*Plasma versus erythrocyte specific activity in the rabbit 21 to 24 hours after injection of radiobromide**

Rabbits	Specific Activity of Plasma	Specific Activity of Erythrocytes	Specific Activity Erythrocytes / Specific Activity Plasma
	cts/min/mEq X 10³	cts/min/mEq X 10³	ratio
3	441 5	449 6	1.02
4	436 3	439 1	1.01
5	143 9	144.8	1.01
6	165 3	160 1	0 97
7	63.19	67.26	1.06
8	58 66	57 70	0 98
9	81 60	78 45	0 96
10	71 47	72 89	1 02
		Mean	1 00
		d	±0 03

* Specific activity refers to the activity of radiobromide per mEq of chloride ion

** $d \quad \sqrt{\frac{\Sigma (x \quad x)^2}{n \quad 1}}$

RESULTS

A Rabbits

The partition of Br^{82} between plasma and erythrocytes was evaluated by determining the S A.R. of these samples in 8 rabbits 21 to 24 hours after the injection of Br^{82} (cf Table I) The mean S.A.R. of $1\ 00 \pm 0\ 03$ indicates that Br^{82} is distributed in direct proportion to chloride across the red cell membrane. These data corroborate previous observations on bromide penetration into red cells (8 14)

Sex-linked differences in body composition have been noted in human subjects in previous studies (10–12 20) Sixteen male-female pairs were studied to evaluate the possibility of sex linked differences in either Cl_e or gut chloride content. Each pair was matched for weight and age and subjected to identical periods of fasting and isotope equilibration Although the Cl_e content of the

TABLE II

*The exchangeable chloride (Cl) and gastrointestinal chloride content of male versus female rabbits**

	Male	Female	t	P
Number	16	16		
Body weight in Kg ± s.d	2 032 ± 0.145	2 030 ± 0.277	0 003	>0 9
Cl_e in mEq ± s d	72 3 ± 8 7	69 0 ± 7 5	1 15	>0 2
Cl_e/body weight in mEq /Kg ± s.d	35 5 ± 3 2	34 2 ± 3 5	1.19	>0 2
**Total G I chloride as % of Cl_e ± s d	17 6 ± 5 4	17 7 ± 5 4	0 052	>0 9

* Each pair of animals was matched for weight and studied after identical equilibration and fasting periods Equilibration periods varied from 21 to 65 hours

** "Total G I Cl_e refers to the intraluminal chloride content of the gastrointestinal tract from the cardia of the stomach to the mid transverse colon

TABLE III

The effect of varying equilibration periods of 21 to 65 hours on the estimated total exchangeable chloride (Cl_e) content in rabbits

Equilibration Period Hours	Number of Animals	Cl_e/Body Weight mEq /Kg (mean ± s d)	t	P
21 - 24	14	33 8 ± 2 92	1.74	>0 10
40 - 48	17	35 7 ± 3 14		
64 - 65	2	36 3*		

* The two values averaged are 35 9 and 36 7 mEq /Kg of body weight Both values fall within one standard deviation of the mean value after either 21 - 24 or 40 - 48 hours of equilibration

male (35 5 ± 3.2 mEq per kgm of body weight) is slightly higher than that of the female (34 2 ± 3 5 mEq per kgm of body weight), neither the differences in Cl_e nor in the "total" gastrointestinal chloride achieves statistical significance (cf Table II) In view of these findings subsequent calculations were made without regard to the sex of the individual animals

To evaluate the possibility of slow penetration of bromide into tissues beyond the accepted 24 hours required for distribution equilibrium, studies on the effect of varying equilibration periods rom 21 to 65 hours on the estimated Cl_e were arried out in 33 rabbits These data are sumnarized in Table III Although the Cl_e per kgm of body weight is slightly higher in the group where 40 to 48 hours were allowed for equilibraion, this difference, 1 9 mEq of chloride per kgm , s not statistically significant ($p > 0\ 10$) These data confirm and extend those reported on the equilibration of bromide and radiochloride (8, 9, 21)

The data tabulated in Tables II and III indicate that sex or prolongation of the period of isotope equilibration may not influence significantly the Cl_e per kgm of body weight The data on these groups have been combined, and the serum chloride and Cl_e are listed in Table IV The serum chloride averaged 98 4 ± 6 4 mEq per liter and the Cl_e averaged 71 0 ± 8 7 mEq , or 34.9 ± 3 5 mEq per kgm of body weight Weir (7) reported a mean Cl_e of 30 2 mEq per kgm of body weight in 10 rabbits estimated by bromide dilution after 1 to 1 5 hours of equilibration, which indicates that 80 to 90 per cent of distribution equilibrium is reached in the first 1 to 2 hours

The penetration of Br^{82} into the gastrointestinal tract has been evaluated by measurements of the

TABLE IV

*The exchangeable chloride (Cl_e) content in the rabbit**

	Body Weight Kg	Serum Chloride mEq /L	Exchangeable Chloride mEq	Exchangeable Chloride mEq./Kg
Mean	2 038	98 4	71 0	34 9
s d	±0 221	±6 4	±8.7	±3 5
Coefficient of variation	10 8%	6 5%	8.2%	10 0 %
Number of animals	33	33	33	

* Equilibration period 21 - 65 hours

TABLE V

The equilibration of Br^{82} with intraluminal gastrointestinal chloride in the rabbit

	No of Animals	Equilibration Period 21-24 hours	No of Animals	Equilibration Period 41-48 hours	t	P
Stomach S.A.R (mean ± s.d)*	6	0 86 ± 0.03	19	0 90 ± 0 02	2 96	<0.01
Small intestine S.A.R (mean ± s.d)	6	0 92 ± 0.03	19	1.00 ± 0.04	3.65	<0.01
Cecum & proximal half of transverse colon S.A.R (mean ± s.d)	6	0 95 ± 0.04	19	0 94 ± 0.06	0	1.00
"Total gastrointestinal contents"** S.A.R (mean ± s.d)	14	0 94 ± 0 06	19	0 94 ± 0 03	0	1.00

* S.A.R specific activity ratio

** "Total gastrointestinal contents" refers to the intraluminal chloride content from the cardia of the stomach to the mid-transverse colon The gastrointestinal contents were pooled and analyzed as a single sample in 8 animals after 21-24 hours of equilibration

S.A.R. of gastric, small bowel and proximal large bowel contents after 21 to 24 hours and 41 to 48 hours of equilibration These data are listed in Table V Equilibration is almost complete in 24 hours but there appears to be some increase in equilibration during the second day in both gastric and small bowel contents ($p < 0\,01$) The S.A.R for stomach may seem to be somewhat less than 100 per cent equilibrated at 48 hours because of volatilization of small amounts of HBr at the time of sample collection Gamble Robertson Hannigan, Foster, and Farr (15) noted more rapid penetration of radiobromide compared with radiochloride into gastric juice of man during the first 2 hours of equilibration. Their data cannot be directly compared with ours because of differences in species and time of sampling Proportional distribution of bromide to chloride between serum and gastric juice has, however been found in patients after chronic bromide ingestion (22)

Data on the effect of short fasting periods on the quantity of intraluminal gastrointestinal chloride relative to Cl_e have been summarized in Table VI There appears to be some decline in intraluminal chloride content during the second fasting day *i e*, total gut chloride of $20\,0 \pm 5\,6$ per cent of Cl_e after 24 hours of fasting versus a value of $16\,0 \pm 4.5$ per cent of Cl_e after 48 hours of fasting ($0\,05 > p > 0\,02$) To be certain of this effect would require more prolonged periods of observation Since there may also be some small gain in distribution equilibrium during the second 24 hours of fasting a 48-hour equilibration and fasting period was used as the basis for measuring intraluminal chloride content.

The amount and distribution of intraluminal chloride after 48-hour equilibration and fasting periods are summarized in Table VII The "total gut chloride is quite significant in quantity, averaging $16\,0 \pm 4.5$ per cent of Cl_e. Gastric

TABLE VI

*The effect of short fasting periods on intraluminal gastrointestinal chloride content**

	Fasting Periods		t	P
	21 27 hours	41 48 hours		
"Total G I chloride % Cl_e (mean ± s.d)	20 0 ± 5 6	16 0 ± 4 5	2 16	<0 05 >0.02
Number of animals	14	17		

* Isotope equilibration period was the same as the fasting period in each case

TABLE VII

*Intraluminal gastrointestinal chloride content in the rabbit**

	Stomach		Small Intestine		Cecum & Proximal Transverse Colon		"Total" G I	
	mEq	% of Cl_e	mEq	% of Cl_e	mEq	% of Cl_e	mEq	% of Cl_e
Mean	8 7	11 7	1 8	2 5	1 2	1 7	11 8	16 0
s d	±3 4	±4 4	±0 4	±0 7	±0 3	±0 4	±3 6	±4 5
Coefficient of variation	39%	38%	22%	28%	25%	24%	31%	28%
Number of animals	17		17		17		17	

* Equilibration and fasting intervals of 41-48 hours, water allowed *ad libitum*

chloride provides the bulk of this quantity, with a mean of 11 7 ± 4 4 per cent of the Cl_e, which is equivalent to 73 per cent of the total gastrointestinal chloride content This is in direct contrast to the distribution of gut sodium and potassium in rabbits, where 72 per cent of gut sodium and 63 per cent of gut potassium are in the cecum and proximal half of the transverse colon (1, 16) The small amount of chloride in the proximal segment of the large bowel, 1 7 ± 0 4 per cent of the Cl_e, suggests efficient cecal conservation of chloride This is borne out by the studies on distal large bowel chloride and the small daily fecal losses of chloride

Table VIII summarizes the data on the intraluminal chloride content of the distal colon and the rates of stool chloride excretion during 24- to 48-hour fasting periods The chloride content of the distal segment of the large bowel is minute, and the fecal chloride excretion rate is quite low, averaging about 1 0 per cent of the Cl_e per day Although chloride exchange between plasma and gut apparently proceeds along the full length of the gastrointestinal tract, it would appear that net flow is in the intraluminal direction at the oral end and in the direction of the blood stream at the aboral end of the gut

B *Human subjects*

The results obtained on post-mortem examination of gut chloride in man are enumerated in Table IX The interval between demise and collection of samples varied from 6 to 22 hours Inspection of these data indicates that there is no correlation between gut chloride content and the post-mortem interval Gastric chloride averaged 14 7 mEq, or 0 9 per cent of the predicted Cl_e, which is approximately 50 per cent of "total" gut chloride (31 7 mEq, or 1 9 per cent of the predicted Cl_e) Most of the remainder was found in the small bowel (11 4 mEq, or 0 7 per cent of the Cl_e) These data, although indicating significantly smaller gut chloride contents in man as compared to rabbits, reveal a similar pattern of

TABLE VIII

The intraluminal chloride content of the distal colon and the rate of stool chloride excretion in rabbits

	Distal Colon and Rectum			Stool Chloride per 24 Hours*		
	mEq	% "Total" G-I Chloride	% Cl_e	mEq	% "Total" G-I Chloride	% Cl_e
Mean	0 06	0 5	0 09	0 73	6 2	1 03
Range	0 02 - 0 13	0 2 - 1 1	0 04 - 0 16	0 - 2 64	0 - 22 4	0 - 3 72
Number of animals	13			13		

* Stool collections were made over a 1 to 2 day period and expressed as chloride excreted per day All animals were fasting during the collection periods

chloride distribution along the length of the gastrointestinal tract. It must be emphasized, however, that these data are not reliable, since agonal or post mortem changes in intraluminal chloride content may have occurred Species differences for intraluminal water content have been reported (23) Definitive measurements in man require access to gut contents immediately after sudden death in previously well individuals

DISCUSSION

Total body chloride estimated *in vivo* in man by isotope dilution averages 31 mEq per kgm of body weight in adult males and 29 mEq per kgm. of body weight in adult females (11, 12, 19) Infants have significantly more chloride averaging 51 mEq per kgm which is to be expected in view of their higher body sodium and water contents (13, 24 25) Weir (7) estimated the Cl_e in rabbits to be 30 mEq per kgm. based on 1- to 1 5 hour bromide dilution. This figure is about 85 per cent of the values of 35 5 mEq per kgm and 34.2 mEq per kgm for male and female rabbits, respectively, with 21 or more hours of equilibration of Br^{82} (cf. Table II)

The distribution of body chloride is important in the interpretation of both metabolic balance data and the movements of water and ions across cell membranes Chloride in the lumen of the gastrointestinal tract is obviously extracellular In man, the concentration of chloride decreases and the concentration of bicarbonate increases progressively from stomach to colon (5 26, 27) The chloride concentration varies from about 150 mEq per liter in the stomach to 50 to 80 mEq per liter in the colon There is a similar pattern of intraluminal chloride distribution in the fasting rabbit and in man studied post mortem The blood to-gut partition of Br^{82} parallels chloride distribution closely as evidenced by the S.A.R. of 0.90 1 00 and 0.94 for stomach, small intestine and proximal large bowel contents respectively (cf Table V) These data support the thesis that intraluminal chloride is an integral part of the body chloride pool

Direct evidence for bidirectional flow of chloride has been obtained by Hogben (3) for the gastric mucosa of the frog and by Visscher and his associates (28–31) for the small bowel mucosa of the dog Gastric transport of chloride is energy dependent and oriented from serosa to mucosa (3) Intestinal transport of chloride probably has an active component as well Bidirectional flux is highest in the jejunum and lowest in the colon (28–31) At the aboral end of the bowel chloride movement is oriented from gut to blood Isotonic chloride solutions placed in the ileum or proximal colon show consistent diminution in chloride concentration and a reciprocal rise in bicarbonate concentration, while the sum of the concentrations of these anions remains unchanged (27 32 33) Taken together, these observations justify the identification of intraluminal chloride as a distinct subdivision of total extracellular chloride.

The rabbit has an impressive amount of intraluminal chloride After 48 hours of fasting 16 per cent of the Cl_e is in the gut and 73 per cent of this quantity, or 11.7 per cent of the Cl_e, is in the stomach (cf Table VII) Since chloride in the proximal colon is only about 1 5 to 2 0 per cent of the Cl_e and the fecal excretion rate is only about 1 0 per cent of the Cl_e per day under fasting conditions intestinal conservation of chloride is clearly an efficient process

Using multiple simultaneous dilution techniques it has been estimated that total intracellular chloride is 30 to 40 per cent of the Cl_e (11 12 19) Since the methods for estimating extracellular fluid exclude gut contents intraluminal chloride is mistakenly included in these intracellular figures. In the rabbit Cl_e averages 35 mEq per kgm , assuming a plasma interstitial compartment of 20 per cent of body weight (34) and a serum chloride concentration of 100 mEq per liter there would be 15 mEq of chloride per kgm of body weight outside of this phase At least 30 per cent of this fraction or 6 mEq per kgm., is intraluminal and less than 25 per cent of the Cl_e is intracellular This does not take into consideration other transcellular fluids so that even this figure is too high

Although post mortem studies on distribution of electrolytes in man are unreliable it is of interest to note the smaller quantities of intraluminal chloride compared to those in the rabbit averaging approximately 2 per cent of the predicted Cl_e (cf Table IX) The pattern of distribution along the length of the gastrointestinal tract is much the same as in rabbits about 50 per cent of total

TABLE IX

Intraluminal gastrointestinal chloride content in the human studied post mortem

Sex	Pathological Diagnosis	Postmortem Interval	Age	Body Weight	Predicted Cl_e	Stomach			Small Intestine			Cecum and Proximal Transverse Colon			"Total" G I		
		hours	yrs	Kg	mEq	mEq	mEq/Kg	% of Cl_e	mEq	mEq/Kg	% of Cl_e	mEq	mEq/Kg	% of Cl_e	mEq	mEq/Kg	% of Cl_e
F 1	Recent myocardial infarction	6	77	35.0	1015	5.9	0.17	0.58	6.9	0.20	0.68	1.1	0.03	0.11	13.9	0.40	1.37
M 2	Rheumatic heart disease with mitral stenosis	11	53	51.8	1601	5.3	0.10	0.33	6.4	0.12	0.40	1.7	0.03	0.10	13.3	0.26	0.83
M 3	Hypertensive cardiovascular disease	22	51	43.6	1347	32.5	0.75	2.42	7.6	0.18	0.57	1.9	0.04	0.14	42.0	0.96	3.12
F 4	Pituitary tumor	9	54	58.2	1688	2.6	0.04	0.15	15.9	0.27	0.94	2.6	0.04	0.15	21.0	0.36	1.25
M 5	Squamous cell carcinoma of lung	7	64	43.6	1264	21.7	0.50	1.72	11.8	0.27	0.93	4.1	0.09	0.32	37.6	0.86	2.97
F 6	Cerebral arteriosclerosis with encephalomalacia	6	56	58.2	1688	14.6	0.25	0.87	5.5	0.10	0.33	10.4	0.18	0.62	30.5	0.53	1.81
F 7	Hypertensive and arterio sclerotic heart disease	22	68	63.4	1839	2.9	0.05	0.16	14.3	0.23	0.78	5.3	0.08	0.29	22.5	0.35	1.22
M 8	Paralysis agitans and bronchopneumonia	16	69	63.2	1953	2.7	0.04	0.14	15.0	0.24	0.77	8.1	0.13	0.41	25.8	0.41	1.32
F 9	Cerebral thrombosis	20	67	65.0	1855	38.4	0.59	2.04	8.7	0.13	0.46	0	0	0	47.1	0.72	2.50
M 10	Dissecting aneurysm of ascending aorta	19	59	68.0	2101	1.9	0.03	0.09	16.2	0.24	0.77	1.2	0.02	0.06	19.3	0.28	0.92
M 11	Recurrent myocardial infarction	16	72	64.5	1993	11.2	0.17	0.56	2.5	0.04	0.13	14.0	0.23	0.70	27.7	0.43	1.39
M 12	Chromophobe adenoma of pituitary	7	50	70.0	2163	24.2	0.35	1.12	21.9	0.31	1.01	7.7	0.11	0.35	53.8	0.77	2.49
M 13	Traumatic demyelinization of cervical spinal cord	12	76	54.5	1684	26.8	0.49	1.59	14.9	0.27	0.88	16.0	0.29	0.95	57.7	1.06	3.43
					Mean	14.7	0.27	0.91	11.4	0.20	0.67	5.7	0.10	0.32	31.7	0.57	1.09
					Range:	1.9 - 38.4	0.03 - 0.75	0.09 - 2.42	2.5 - 21.9	0.04 - 0.31	0.13 - 1.01	0 - 16.0	0 - 0.29	0 - 0.95	13.3 - 57.7	0.2[illegible] - [illegible]	0.83 - 3.43

gastrointestinal chloride in the stomach, 35 per cent in the small bowel, and 15 per cent in the proximal half of the large bowel The validity of these observations is not yet established however for the previously stated reasons

SUMMARY

Intraluminal gastrointestinal chloride content was measured in rabbits and in human subjects studied post mortem In the former, gut chloride was referred to Cl_e estimated with KBr^{82} while in the latter gut chloride was referred to the predicted Cl_e values.

Total exchangeable chloride averaged 34.9 ± 3.5 mEq per kgm of body weight in rabbits Of this 16.0 ± 4.5 per cent was in the lumen of the gastrointestinal tract with 11.7 ± 4.4 per cent in the stomach 2.5 ± 0.7 per cent in the small intestine, and 1.7 ± 0.4 per cent in the cecum and proximal half of the large intestine Radiobromide exchange equilibrium was complete to within 10 per cent for all segments of the gastrointestinal tract 48 hours after injection No significant difference in either the Cl_e or the quantity of intraluminal gastrointestinal chloride was found between male and female rabbits

Human subjects at post mortem examination had relatively small amounts of intraluminal gastrointestinal chloride the mean values were 1.9 per cent of the predicted Cl_e in the total gastrointestinal tract, with 0.91 per cent in the stomach 0.67 per cent in the small intestine and 0.32 per cent in the cecum and proximal transverse colon The quantity of intraluminal chloride in normal man cannot be reliably inferred from these data.

The implications of these data are discussed in terms of the dynamics of chloride transport across the gastrointestinal mucosa and the anatomy of body chloride.

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GASTROINTESTINAL WATER AND ELECTROLYTES IV THE EQUILIBRATION OF DEUTERIUM OXIDE (D_2O) IN GASTROINTESTINAL CONTENTS AND THE PROPORTION OF TOTAL BODY WATER (T.B W) IN THE GASTROINTESTINAL TRACT[1]

By F GOTCH,[2] J NADELL,[3] AND I. S. EDELMAN[4]

(From the Department of Medicine University of California School of Medicine and the San Francisco Hospital San Francisco Calif)

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The concept of the anatomy of body water distribution as a two-compartment system consisting of intracellular and extracellular fluid has been shown to be inadequate (1–5) The heterogeneous nature of the extracellular fluid compartment has been established by previous studies on bone (2), dense connective tissue (6) and transcellular fluid (3–5) Cizek (3) has demonstrated that intraluminal gut water is a significant subdivision of body water in a number of species

Neglecting the contribution of transcellular fluid to body water results in considerable errors in the derived normal values for body water compartments Furthermore, transcellular fluid if large enough in volume, must be considered as potentially important in determining the volume and osmolarity of plasma, interstitial and intracellular fluid by ion and water flux in or out of transcellular pools in response to metabolic stimuli

In the preceding three papers of this series we reported the measured amount of sodium potassium and chloride contained within the lumen of the gut in rabbits and in human subjects at post mortem (4 5 7) The present study is similar in design to these previous experiments and presents observations on a) the amount of intraluminal gastrointestinal water expressed as a fraction of total body water (T B W) and the extent of deuterium oxide (D_2O) exchange equilibrium in gut contents in rabbits, and b) the amount of intraluminal water in man at post mortem examination.

METHODS

A Rabbits

Forty adult albino rabbits were studied in pairs consisting of a male and a non-gravid female. The animals were fasted and thirsted. All urine passed during the period of isotope equilibration was collected in a metabolism cage. This period varied from 2 to 5 hours. Each animal was injected intraperitoneally with 2 ml. of D_2O[5] from a calibrated syringe. At the end of the equilibration period each animal was anesthetized with 2 ml. of 2 per cent sodium pentobarbital injected into a dorsal ear vein and was then weighed to the nearest gram. A blood sample was obtained at this time by cardiac puncture through the intact chest wall with a syringe containing dry heparin. The syringe was capped and centrifuged immediately after collection and the separated plasma was aspirated, sealed in a glass ampoule and stored in a freezer

The gastrointestinal tract was removed in three segments by cutting between double ligatures placed at the cardia of the stomach the pylorus the ileocecal valve and at a position in the transverse colon where there was a transition point between semi solid and solid stool pellets. After removal each segment was washed with distilled water dried with towels and weighed to the nearest gram.

The contents of each segment were milked into one ligated end, a small incision was made, and an aliquot of contents was expressed into a dried test tube, which was quickly stoppered and centrifuged. The supernatant was then aspirated and sealed in a glass ampoule and stored in a freezer Each segment was then opened longitudinally and the remaining contents were evacuated into a clean container by gently stripping and then washing the mucosal surface with distilled water The

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[2] Research Fellow of the American Heart Association.

[3] Research Fellow of the National Heart Institute of the United States Public Health Service.

[4] Established Investigator of the American Heart Association.

[5] Deuterium oxide, 99 6 per cent pure was obtained from Abbott Laboratories as a sterile isotonic saline solution.

TABLE IV

The equilibration of D_2O between plasma and gastrointestinal contents

Equilibration time (hours)	Stomach				Small intestine				Cecum and transverse colon			
	No of animals	S.A.R. mean ± s.d	t*	p	No of animals	S.A.R. mean ± s d	t*	p	No of animals	S.A.R. mean ± s d	t*	p
2	10	0.86 ± .08	5.60	<0.001	9	0.95 ± .05	2.94	<0.02 >0.01	8	1.00 ± .09	0	1.0
3	9	0.93 ± .07	3.04	<0.02 >0.01	9	0.99 ± .03	1.00	>0.3	8	1.01 ± .04	.71	0.5
4	10	0.98 ± .05	1.25	>0.2	10	1.00 ± .03	0	1.0	10	1.01 ± .03	1.11	0.3
5	10	1.01 ± .02	1.67	>0.1	10	1.02 ± .03	2.22	>0.05 <0.10	10	1.01 ± .03	1.11	0.3

$$^{*}t = \frac{1.00 - \bar{x}}{\frac{s\,d}{\sqrt{n}}}$$

Human subjects

The volumes of measured intraluminal gastrointestinal water expressed in terms of predicted T B W at post mortem in 13 human subjects are presented in Table V "Total" gastrointestinal water comprised 1.4 per cent of the predicted T B W The stomach, small intestine and the proximal half of the large intestine were found to contain an average of 0.4, 0.7 and 0.3 per cent of predicted T B W, respectively "Total" intraluminal water content varied only from 0.5 to 2.2 per cent of T B W In contrast, the intraluminal pool of the rabbit comprised 12 per cent of T B W

DISCUSSION

The purpose of these experiments was to study the magnitude and exchange characteristics of intraluminal gastrointestinal water

Total body water and the volume of intraluminal water in healthy animals might be expected to vary with body weight, age, sex and duration of fasting and thirsting The animals studied were all young adults, each weighing about 2 Kg Total body water was approximately 75 per cent of body weight in both male and female rabbits The absence of a difference in T B W between sexes contrasts with the significantly higher total body water content in males noted in studies on man (10) It is likely that these findings are explained by the fact that the female rabbits were all young nulliparous adults Prepubertal human females have been shown not to differ from male subjects in body water content (10, 12) Cizek (3), in studies of somewhat larger and older rabbits, did find a significantly higher total body water in male than in female rabbits

The volume of intraluminal water was not affected by the sex of the animal nor by fasting and thirsting up to 4 hours The magnitude of this transcellular pool was approximately 12 per cent of T B W, which corresponds well with previous measurements (3) and represents a large fraction of the body water content of this species This volume is comparable to one-half the volume of interstitial fluid or to twice the volume of plasma (13) The size of this subdivision of body water raises the possibility that it may contribute significantly to changes in plasma-interstitial fluid volumes induced by physiological or pathological influences Furthermore, calculations of the distribution of water and ions which are based on a more simplified concept of the anatomy of body water, *i e*, a two-compartment system, will be erroneous in proportion to the volume of transcellular fluid in the species under study

The observation that the S A R. of gut water to plasma water reaches unity in all segments within 4 hours indicates that this transcellular pool of water is in exchange equilibrium with the remainder of T B W D_2O exchange is fastest in large bowel contents, while slower exchange occurs in small bowel contents, and the slowest exchange occurs in stomach water If this isotope penetrated stomach mucosa only and then passed down the intestinal tract, water in the large bowel should equilibrate last Our data effectively exclude this possibility and suggest instead that water penetrates across the mucosa of the gut throughout the length of the tract The delay of

TABLE V

Intraluminal gastrointestinal water content in the human studied post mortem

Sex	Pathological diagnosis	Post mortem interval (*hours*)	Age (*yrs.*)	Body weight (*Kg*)	Predicted T.B.W (*liters*)	Stomach (*ml.*)	Stomach (*ml./Kg.*)	Stomach (*% T.B.W.*)	Small intestine (*ml.*)	Small intestine (*ml./Kg.*)	Small intestine (*% T.B.W.*)	Cecum and proximal transverse colon (*ml.*)	Cecum and proximal transverse colon (*ml./Kg.*)	Cecum and proximal transverse colon (*% T.B.W.*)	"Total" G.I. (*ml.*)	"Total" G.I. (*ml./Kg.*)	"Total" G.I. (*% T.B.W.*)
F 1	Recent myocardial infarction	6	77	35.0	22.4	31	1.5	0.3	100	2.9	0.5	13	0.4	0.1	164	4.8	0.8
M 2	Rheumatic heart disease with mitral stenosis	11	53	51.8	34.4	65	1.3	0.2	75	1.5	0.2	44	0.9	0.1	184	3.7	0.5
M 3	Hypertensive cardiovascular disease	22	31	43.6	29.7	233	3.4	0.8	335	7.7	1.1	33	0.8	0.1	601	13.9	2.0
F-4	Pituitary tumor	9	54	58.2	26.8	32	0.6	0.1	202	3.5	0.8	60	1.0	0.2	294	5.1	1.1
M 3	Squamous cell carcinoma of lung	7	64	43.6	29.7	233	3.4	0.8	131	3.5	0.8	13	0.3	0.1	397	9.2	1.7
F-6	Cerebral arteriosclerosis with encephalomalacia	6	56	58.2	26.8	132	2.3	0.5	60	1.0	0.2	178	3.1	0.7	370	6.4	1.4
F 7	Hypertensive and arteriosclerotic heart disease	22	68	63.4	28.0	11	0.2	0.1	234	3.7	0.8	80	1.3	0.3	325	3.2	1.2
M-8	Paralysis agitans and bronchopneumonia	16	69	63.3	37.7	64	1.0	0.2	349	5.5	0.9	430	6.8	1.1	843	13.3	2.2
F-9	Cerebral thrombosis	20	67	65.0	29.9	226	3.5	0.8	95	1.5	0.3	—	—	—	321	5.0	1.1
M 10	Dissecting aneurysm of ascending aorta	19	59	68.0	38.0	34	0.5	0.1	241	3.3	0.6	20	0.3	0.1	293	4.3	0.8
M 11	Recurrent myocardial infarction	16	72	64.5	38.0	97	1.5	0.3	352	5.5	0.9	15	0.2	0.1	464	7.2	1.3
M 12	Chromophobe adenoma of pituitary	7	50	70.0	37.3	170	2.4	0.5	220	3.1	0.6	107	1.5	0.3	497	7.0	1.4
M 13	Traumatic demyelinization of cervical spinal cord	12	76	54.5	38.6	181	3.3	0.5	345	6.3	0.9	7	0.1	0.1	533	9.9	1.5
					Means:	118	2.2	0.4	206	3.8	0.7	83	1.4	0.3	407	7.4	1.4
					Ranges:	11–233	0.2–5.4	0.1–1.2	60–352	1.0–7.7	0.2–1.1	7–430	0.1–6.8	0.1–1.1	164–843	3.6–13.8	0.5–2.2

TABLE VI

Summary of intraluminal gastrointestinal sodium, potassium, chloride, and water content in the rabbit

	Sodium		Potassium		Chloride		Water		Calculated concentration in intraluminal water*		
	M ± s d (*mEq*)	M ± s d (*% Na_e*)	M ± s d (*mEq*)	M ± s d (*% K_e*)	M ± s d (*mEq*)	M ± s d (*% Cl*)	M ± s d (*ml*)	M ± s d (*% T B W*)	Sodium (*mEq/L.*)	Potassium (*mEq/L.*)	Chloride (*mEq/L.*)
Stomach	0 8 ±0 4	0 9 ±0 4	0 7 ±0 3	0 7 ±0 3	8 7 ±3 4	11 7 ±4 4	65 ±15	4 1 ±0 9	12	11	134
Small intestine	3 1 ±1 1	3 2 ±1 2	1 2 ±0 4	1 2 ±0 4	1 8 ±0 4	2 5 ±0 7	31 ±12	2 0 ±1 0	100	39	58
Cecum and transverse colon	10 0 ±2 0	10 2 ±2 1	4 5 ±3 1	4 5 ±2 7	1 2 ±0 3	1 7 ±0 4	93 ±22	6 0 ±1 6	108	48	13
"Total" G I content	13 7 ±2 4	14 2 ±2 4	7 1 ±2 8	7 2 ±2 2	11 8 ±3 6	16 0 ±4 5	189 ±40	12 1 ±2 7			

* Calculated concentrations are derived by dividing intraluminal sodium, potassium, and chloride contents by intraluminal water content Each quantity was determined in separate series of animals

equilibration in stomach water, where 4 hours was required for distribution equilibrium compared with 1 and 2 hours for colon and small bowel, respectively, may be a result of at least three factors The ratio of membrane surface area to intraluminal volume may be smaller in stomach than in either large or small bowel The ratio of surface to volume has been proposed as the basis for D_2O exchange rates in other transcellular pools (14) The delay of equilibration of labelled water in small bowel contents compared to large bowel contents cannot, however, be explained on comparative ratios of surface area to volume A second possible explanation is that mucosal blood flow, and consequently the rate of delivery of isotope in proportion to the volume of intraluminal water, may be highest in the large bowel and least in the stomach (15) Finally, active transport of water across gut mucosa may occur and account for some of these differences (16)

In the course of these studies it was noted that the contents of the cecum and the proximal transverse colon were semiliquid and that in the mid-transverse colon there was a sharp transition zone, 1 to 2 cm in length, where the contents were transformed into hard, dry pellets of stool This would suggest that the mucosa of the mid-transverse colon acts to conserve water efficiently

Data from previous experiments in which the intraluminal content of sodium, potassium, and chloride were determined are summarized in Table VI (4, 5, 7) The last three columns in Table VI show the calculated concentrations for each of these ions in intraluminal water of stomach, small bowel and large bowel It is apparent from these values that the concentration of sodium, potassium and chloride maintained in intraluminal water bears no direct relation to the electrolyte structure of extracellular fluid It would seem that their concentration and abundance in intraluminal water are determined by autonomous mechanisms in the gastrointestinal tract

The presence of 14 per cent of Na_e, 7 per cent of K_e and 16 per cent of Cl_e in the contents of the gastrointestinal tract in rabbits has important implications in body partition studies where the normal anatomy of ion distribution or of ion shifts is measured (4, 5, 7) Calculating the extracellular fluid volume from a chloride space and assuming that all chloride exists in the same concentration as in plasma will lead to significant errors Changes in the extracellular space inferred from changes in chloride concentration in plasma and external chloride balance may also be misleading since these calculations are based on the assumption that all, or nearly all, of the body chloride is in the plasma-interstitial fluid volume

The volume of intraluminal water found in the gastrointestinal tract of man was a much smaller fraction of T B W than in the rabbit The significance of this species difference cannot be determined from our data since the observations on

human subjects must be evaluated cautiously for several reasons Total body water was predicted from data on normal subjects, in contrast, accurate measurements were made in the rabbits Significant migration of water from the gut may occur in critically ill patients. Post-mortem changes in intraluminal volume may have taken place during the 6 to 22 hours that elapsed between death and autopsy in these subjects. The measurements made in the human subjects consequently are not reliable and further studies are needed to establish the amounts of intraluminal water and electrolytes in normal man.

SUMMARY

The volume of intraluminal gastrointestinal water was measured in rabbits and in human subjects studied post mortem In rabbits this volume was referred to T.B W as determined by D_2O dilution. In man the intraluminal gut water was referred to predicted T.B W values

Total body water averaged 75 per cent of the body weight in rabbits, 12 per cent of T B W was contained in the lumen of the "total" gastrointestinal tract, with 4 per cent in the stomach 2 per cent in the small intestine and 6 per cent in the large intestine. No significant difference between sexes was noted in either total body water or the volume of intraluminal gut water Deuterium oxide equilibration was complete in large bowel water and nearly complete in small bowel water in 2 hours but required 4 hours for completion in stomach water The significance of delayed D_2O equilibration in stomach water compared with more distal segments of bowel was discussed with respect to the sites and mechanisms of D_2O exchange across gastrointestinal membranes

The gastrointestinal tract of man at post mortem examination contained approximately 1 5 per cent of the predicted T.B W The mean values were 0 4 per cent for stomach, 0.7 per cent for small bowel and 0.3 per cent for proximal large bowel These values cannot be considered to represent the volume of intraluminal gut water to be found in the normal living human subject.

The amounts of intraluminal gut sodium potassium, chloride and water in the rabbit are summarized in tabular form.

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THE CHEMICAL ESTIMATION OF ACYL GLUCURONIDES AND ITS APPLICATION TO STUDIES ON THE METABOLISM OF BENZOATE AND SALICYLATE IN MAN

By DAVID SCHACHTER[1]

(*From the Department of Pharmacology-Biochemistry School of Aviation Medicine USAF Randolph Air Force Base Tex*)

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Structurally, glucuronides are the condensation products of hydroxyl bearing compounds with the first hemiacetal carbon of D-glucuronic acid Two groups have been described (1) Alcohols and phenols form *ethereal* glucuronides, which are resistant to hydrolysis by mild alkali and do not reduce alkaline copper reagents. Carboxylic acids form *ester* glucuronides which are easily split by mild alkali to liberate free glucuronic acid a reducing agent. The term *acyl glucuronide* is introduced here for members of the latter group to denote clearly conjugation via the carboxyl group

Another chemical reaction characteristic of the acyl glucuronides is described in this report. At room temperature and neutral pH the acyl group can be transferred to hydroxylamine to form characteristic hydroxamic acids (Reaction I)

$$R{-}CO{-}O{-}CH{-}(CHOH)_3{-}CH{-}COOH + NH_2OH \rightarrow$$

$$R{-}CO{-}NH{-}OH + HO{-}CH{-}(CHOH)_3{-}CH{-}COOH$$

On addition of acid ferric chloride solution hydroxamic acids yield colored products which can be measured spectrophotometrically (2) Thus, conversion to the stable hydroxamates provides a sensitive, chemical method for estimating the relatively unstable acyl glucuronides The derivatives are easily extracted into organic solvents and can be identified by chromatographic and other techniques Non acyl glucuronides do not form hydroxamic acids

The methods reported here have permitted more extensive studies on the metabolic fate of aromatic acids in man Since urinary glucuronides have been found following the ingestion of benzoate, salicylate, and probenecid (1, 3), observations were made on the metabolism of these clinically important drugs

METHODS AND MATERIALS

Drug experiments The subject was a healthy 28-year-old white male, weighing 60 kilos. His diet was not rigidly controlled, but remained fairly uniform throughout the period of investigation all experiments were begun in the fasting state. At least 2 weeks elapsed between successive doses of drug On the morning of an experiment, a control urine was collected over a period of several hours and the appropriate drug was ingested as a solution of its sodium salt. Subsequently timed voided urine samples were tested immediately for acyl glucuronides and stored at 5 C until completion of the remaining estimations. When necessary the excretory rates of urinary drug metabolites were corrected for pre-ingestion, endogenous rates

Estimation of acyl glucuronides These compounds are estimated by conversion to their hydroxamates, using modifications of the procedure of Lipmann and Tuttle (2) The pH of the neutralized hydroxylamine reagent is critical and should be 7 0 ± 0.2. Stock solutions of NaOH (14 per cent) and hydroxylamine hydrochloride (28 per cent) are titrated against each other with a Beckman glass electrode pH meter to determine the exact proportions required. Urine samples containing 0.5 to 2.0 μmoles of acyl glucuronide are incubated with 0.5 ml. of freshly prepared neutral hydroxylamine for 2 hours at room temperature before color development and comparison with authentic hydroxamate standards. Figure 1 demonstrates the complete conversion of benzoyl glucuronide to benzoyl hydroxamate under these conditions.

[1] Present address Department of Medicine, College of Physicians and Surgeons, New York City N Y

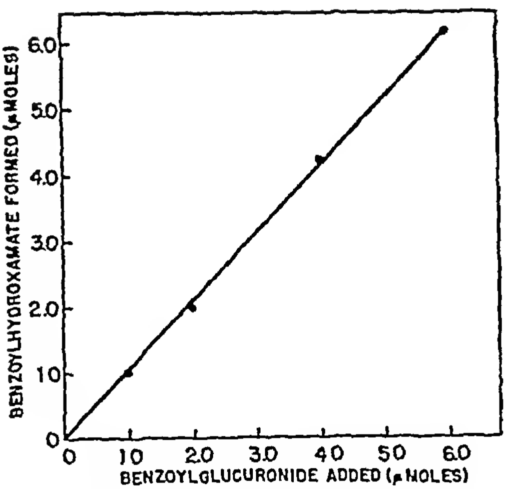

FIG 1 QUANTITATIVE CONVERSION OF BENZOYL GLUCURONIDE TO BENZOYL HYDROXAMATE

The indicated amounts of crystalline benzoyl glucuronide in 0.5 ml water were mixed with 0.5 ml neutral hydroxylamine and incubated 2 hours at room temperature. The volume was adjusted to 2 0 ml with water, and 0.5 ml of 3 N HCl followed by 0.5 ml of 5 per cent $FeCl_3 \cdot 6H_2O$ in 0 1 N HCl added. The optical density at 540 mµ was compared with that of a benzoyl hydroxamate standard.

To estimate salicyl acyl glucuronide (SAG) in urine by this procedure, 6 N HCl is substituted for 3 N HCl to minimize the formation of purple products arising from salicylate and salicylurate. Further, a correction is made in each estimation by subtracting the optical density at 540 mµ of a similarly treated, hydroxylamine free control[2]

The specificity of hydroxamate formation was studied with a series of 9 glucuronides, using 2 0 µmoles of each in the assay (The conditions are described in the legend to Figure 1) Only benzoyl- and o-methoxybenzoyl glucuronides yielded such products, and no reaction was observed with the glucuronides of salicylamide, N-acetyl salicylohydrazine, 3-hydroxycoumarin, pregnandiol, phenolphthalein, menthol, and borneol

Identification of urinary acyl glucuronides Urinary hydroxamate-forming materials appear after the administration of benzoate and salicylate. The following evidence identifies these as benzoyl- and salicyl acyl glucuronides (SAG), respectively These compounds are completely hydrolyzed by 0 1 N NaOH in 10 minutes at room temperature, and 84 per cent hydrolyzed by incubation with bacterial β-glucuronidase (250 units per ml) for 60 minutes at 38° C A single hydroxamate spot was detected for each compound by paper chromatography of urinary aliquots, using Whatman No 1 paper and the ascending technique, with water-saturated n butanol glacial acetic acid (80 20, v/v) as the solvent system. These spots reduce aniline phthalate (4), and give a positive carbazole reaction for hexuronic acid (5) after elution into water The benzoate metabolite moves at a rate (Rf 0 67) similar to that of crystalline benzoyl glucuronide. The salicylate conjugate (Rf 0.72) liberates free salicylate on hydrolysis in 6 N HCl at 100° C for 1 hour

The hydroxamate derivatives of these urinary metabolites were extracted at neutral pH into ether and chromatographed on paper as described above, with water-saturated n-butanol as the developing solvent. Movement of the benzoyl derivative (Rf 0 79) corresponded to that of an authentic sample of benzoyl hydroxamate, and that of the salicyl compound (Rf 0 85) to crystalline salicyl hydroxamate.

That the urinary SAG is a monoglucuronide has been established in the following manner its hydroxamate is completely extractable into ether, while that of the diglucuronide would be ether-insoluble. In addition, the hydroxamate is chromatographically homogeneous and identical in behavior with synthetic salicyl hydroxamate.

Estimation of urinary salicyl metabolites In addition to SAG, salicyl phenolic glucuronide (SPG), salicylurate, and total salicyl were estimated.

SPG accounts for the difference between the quantity of SAG and the total salicyl glucuronide excreted The latter is estimated by the salicylate liberated by bacterial β-glucuronidase. An aliquot of urine, adjusted to pH 2, is extracted twice with an equal volume of ether to remove salicylate and salicylurate, and readjusted to pH 6 0 Samples are withheld for initial salicylate (6) and SAG determinations Five hundred units of β-glucuronidase are added to each ml. of extracted urine, the mixture is incubated at 38° C for 8 hours, an identical amount of enzyme again added and the incubation repeated.[3] Finally, 10 per cent perchloric acid filtrates are prepared and the free salicylate concentrations again determined.

$$\text{SPG} = \text{final salicylate} - \text{initial salicylate} - \text{SAG}$$

Salicylurate is estimated by a paper chromatographic technique. Suitable volumes of urine are quantitatively applied in duplicate to Whatman No 1 paper for ascend-

[2] A satisfactory method of eliminating blank values due to salicylate and salicylurate is to extract the reaction mixture after formation of salicyl hydroxamate with 4 volumes of ether at neutral pH. Salicylate and salicylurate remain behind, while over 90 per cent of the hydroxamate is extracted. An aliquot of the ether is evaporated and color developed with HCl and ferric chloride. Values for acyl glucuronide in salicyl urine were identical, when estimated by both this procedure and that described in the text.

[3] Enzyme-treated urine was further hydrolyzed in 6 N HCl at 100° C for 3 hours, and free salicylate again estimated. The results indicated that enzymatic hydrolysis was at least 80 per cent complete under the conditions described in the text. Enzymatic hydrolysis is preferable to acid hydrolysis since specificity for glucuronides is obtained

ing chromatography with n butanol ethanol (40 11 v/v) saturated with an ammonium carbonate buffer (7) as the solvent system. The dried paper chromatograms are viewed with an ultra violet lamp and the fluorescent salicylurate areas (Rf 0.31) outlined and eluted into water (The salicylate fluorescent area corresponds to Rf 0 66.) To 2.8 ml. of eluate, containing about 0.5 µmole salicylurate, 0.2 ml. of 0 1 M ferric chloride in 0 07 N HCl is added. The optical density of the purple products is measured at 540 mµ in the Beckman model B spectrophotometer Recoveries of known quantities of salicylurate, chromatographed simultaneously have ranged from 90 0 to 102.0 per cent.

The procedure of Lester Lolli and Greenberg (6) was followed in estimating total urinary salicyl.[4]

Corrected total salicyl = observed total salicyl + salicylurate × 0 676 × 0.225 + salicylurate × 0.676 × 0.775 − salicylurate × 0 676 × 0.775 × 0.84

Corrected total salicyl = observed total salicyl + salicylurate × 0.236. The salicylurate concentration is estimated by the chromatographic method described in the text.

Other methods. Chemical estimations were employed for hippurate (9) hexuronic acid (5) and probenecid (3) Probenecid conjugates were hydrolyzed by refluxing in 4 0 N H_2SO_4 for 60 minutes. Optical density measurements were made in the Beckman model B spectrophotometer

Materials The following compounds were prepared according to published methods and recrystallized from hot water benzoyl glucuronide (10) m.p 181–2° (decomp) (Pryde and Williams 183 [11]) salicyluric acid (12) m.p. 166 (Quick 167 [12]) salicyl hydroxamic acid (13) m.p. 168 (Jeanrenaud 168° [13]) A reference standard solution of benzoyl hydroxamate was prepared by reaction of neutral hydroxylamine with recrystallized benzoic anhydride (14) Crystalline salicylamide glucuronide (174) o-methoxybenzoyl glucuronide (123–4) and N-acetyl salicylohydrazine glucuronide (208–10) were gifts from Doctor R. T Williams to Captain R. M. Dowben 3 hydroxycoumarin glucuronide (207–8) was a gift from Captain R. M Dowben menthol borneol pregnandiol and phenolphthalein glucuronides were purchased from the Sigma Chemical Company as was the bacterial β-glucuronidase.

[4] In this procedure aliquots of salicyl urine are hydrolyzed to liberate salicylic acid, which is extracted into ether and estimated by reaction with ferric chloride. The "total salicyl concentration so measured requires complete hydrolysis of all salicyl conjugates. However 67 6 per cent of the glycine conjugate remains unhydrolyzed, as determined by the liberation of glycine (8) in similarly treated salicyluric acid solutions. Hence it is necessary to correct the observed total salicyl concentration for 1) incomplete ether extraction of salicylurate (77.5 per cent as compared to 100 per cent for salicylate) and 2) relative intensity of the colored products formed with ferric chloride (84 0 per cent for salicylurate as compared to salicylate)

RESULTS

Ingestion of benzoate

In individual experiments, the subject was fed 6.9, 13.9 34.7, and 69.3 millimoles of sodium benzoate.[5] Complete elimination of the drug as urinary hippurate and benzoyl glucuronide ensued in each instance, with final recoveries of 98 to 104 per cent. The relative amounts of benzoate conjugated with glycine and with glucuronic acid varied with the dosage, as shown in Figure 2 The glucuronide moiety increased progressively from 0 4 per cent (0 03 millimoles) to 3 0 per cent (2 08 millimoles) Hippurate accounted for the bulk of conjugated benzoate throughout this dose range.

The cumulative urinary recovery of each metabolite is plotted in Figure 3 for a representative experiment. The recoveries of acyl glucuronide and hippurate ran a parallel course, a characteristic observed at all the dosage levels examined. Complete excretion of benzoate required 3 to 4 hours after the two smaller doses and 10 to 14 hours after the larger amounts

As indicated in Table I, the maximal urinary excretory rate (µmoles per minute) achieved in a given experiment by either metabolite depended on the dose of benzoate Limiting values for hip-

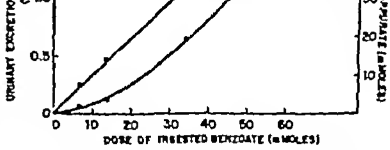

FIG. 2. QUANTITIES OF URINARY HIPPURATE AND BENZOYL GLUCURONIDE RECOVERED AFTER VARIOUS DOSES OF BENZOATE

The right and left hand vertical scales refer to hippurate and benzoyl glucuronide, respectively

[5] Corresponding to 1.0 2.0 5.0 and 10 0 grams

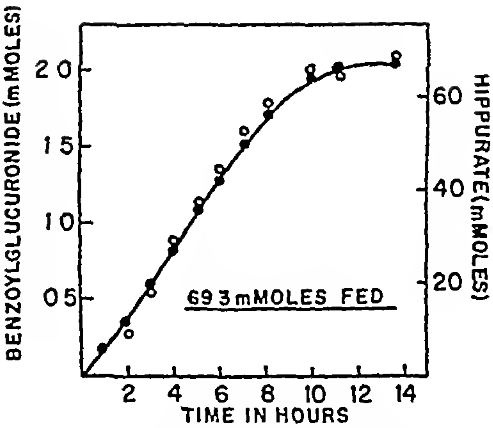

FIG 3 CUMULATIVE URINARY RECOVERIES OF HIPPURATE (—●—●—) AND BENZOYL GLUCURONIDE (O)

purate excretion were approached at a dose of 13 9 millimoles Quick (15) has demonstrated that limitations in the availability of glycine may account for this, and the peak rate attained in these experiments (130 5 μmoles per minute) is within his range for maximal rates of glycine mobilization in man (122 to 155 μmoles per minute) By contrast, the maximal excretory rate of benzoyl glucuronide was approximately proportional to the benzoate dose throughout the range studied It is significant that the glucuronide was

TABLE I

Maximal rates of hippurate and benzoyl glucuronide excretion following various doses of benzoate

Dose of sodium benzoate		Maximal urinary excretory rate (μmoles per min)	
(grams)	*(millimoles)*	Hippurate	Benzoyl glucuronide
1 0	6 9	54 8	0 4
2 0	13 9	108 2	1 0
5 0	34 7	111 3	2 6
10 0	69 3	130 5	5 5

detected at a dosage level (6 9 millimoles) far below that required for maximal hippurate excretion These observations fail to support the supposition (15) that the glucuronide synthesis is a reserve detoxication mechanism, operating only when the glycine supply is exceeded Further pertinent evidence is obtained from the data in Figure 3 After the seventh hour in this experiment, the excretion of hippurate was well below its maximal rate, yet significant benzoyl glucuronide excretion persisted

Ingestion of salicylate

Four experiments were performed, the subject ingesting 7 5, 15 0, 22 5, and 30 0 millimoles of sodium salicylate [6] The relative quantities of urinary salicyl metabolites excreted after these doses are listed in Table II With doses of 15 0 to 30 0 millimoles about 50 per cent of the amount fed was recovered as urinary salicyl in 24 hours (and 85 per cent at the end of 48 hours) Salicylurate made up 60 per cent of this, and the glucuronides 30 per cent, values which agree closely with previous observations (12, 16) SAG and SPG were present in about equal amounts Cumulative urinary recoveries of the salicyl metabolites, in a representative experiment, are depicted in Figure 4 Whereas total salicyl, salicylurate, and SAG ran a course parallel to each other with time, the recovery of SPG was relatively lower in the early hours and higher thereafter The maximal rates of excretion (μmoles per minute) of the urinary products varied with the doses, as shown in Table II, limiting values for these rates were not achieved in this range

Characteristic differences in the excretory patterns of SAG and SPG were observed in all the

[6] Corresponding to 1 0, 2 1 3 1, and 4.2 grams

TABLE II

Urinary metabolites of salicylate following various oral doses

Dose *(millimoles)*	Urinary salicyl recovered in 24 hours, *per cent of dose*	Urinary metabolites recovered in 24 hours per cent of total urinary salicyl			Maximal rates of urinary excretion (μmoles per min)			
		Salicyl urate	SAG	SPG	Total salicyl	Salicyl urate	SAG	SPG
7 5	78 6	64.5	13 1	12 7	4 5	3 0	0 8	0 6
15 0	54 5	62 8	14 8	21 0	5 6	4 1	1 0	1 4
22 5	49 4	74 0	14 3	15 1	8 0	6 0	1 5	1 5
30 0	50 8	59 6	16 4	12 1	11 1	6 8	1 9	1 7

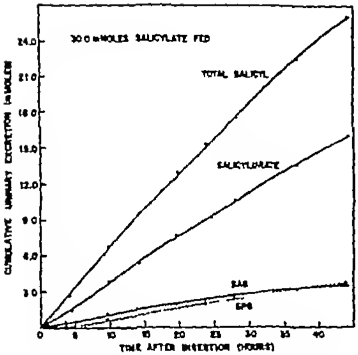

FIG. 4 CUMULATIVE URINARY RECOVERIES OF SALICYL METABOLITES

experiments These are illustrated in Figure 5 The peak SAG excretory rates were observed within 5 to 10 hours after ingestion, with a rapid fall thereafter By contrast the SPG peak was obtained after 20 to 30 hours and the subsequent fall-off was more gradual

Ingestion of probenecid

Seven and one-half millimoles (2 1 grams) of this drug (sodium p-(dipropylsulfamyl) benzoate) were administered to the subject. At the end of the first, second third and fourth days, the cumulative urinary recoveries were 35 1, 59 1, 73.8 and 79 1 per cent. Consistently, 80 per cent appeared as the acyl glucuronide. The excretion of total hexuronic acid, estimated by the carbazole method was equivalent to that of acyl glucuronide. By contrast, the urinary hexuronic acid concentration following benzoate was over three times that of benzoyl glucuronide, and after salicylate twice as much as that of the combined glucuronides It thus seems likely that probenecid glucuronide is much less susceptible to hydrolysis in the body than the glucuronides of benzoate and salicylate.

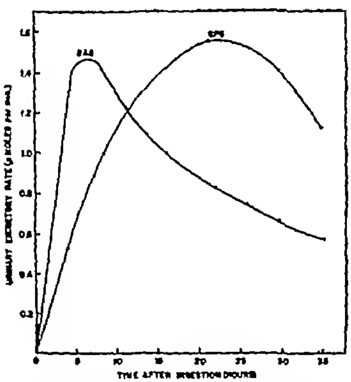

FIG. 5 RATES OF URINARY EXCRETION OF SAG AND SPG AFTER INGESTION OF SALICYLATE

DISCUSSION

Previous investigators have described the transfer to hydroxylamine of acyl groups linked to acids as anhydrides (2) or to alcohols as esters (17) It is of interest, therefore, that the acyl glucuronides, which constitute an intermediate category with acyl groups linked to a hemiacetal bearing carbon, also form hydroxamates This provides a sensitive method for their chemical estimation, and further characterizes them as a unique group within the class of glucuronides

Conversion to the stable hydroxamates offers the advantage of a trapping mechanism for the readily hydrolyzed acyl glucuronides These hydroxamates are readily extracted purified and subsequently identified. As a differential reaction, the hydroxamate method allows separate estimation of acyl and non acyl glucuronides in mixtures of both. These several advantages made possible the observations described in this report.

Excretion of benzoyl glucuronide could be detected following the administration of as little as 6.9 millimoles of benzoate, although several times this amount has been required in the past (15) Thus it could be demonstrated that the glucuronide synthesis occurs independently whether or not glycine conjugation is maximal Only a minimal estimate of the actual rate of benzoyl glucuronide synthesis is obtained from the urinary recovery as noted by Quick (15) The extent to which this conjugate may be broken down in the body is suggested by the three fold excess of urinary hexuronic acid relative to benzoyl glucuronide. Therefore it seems inappropriate to designate benzoyl glucuronide formation as merely a spare detoxication mechanism

method of Van Slyke (10), and concentration of sodium and potassium in red blood cells separated from plasma after one-half hour centrifuging at 3,000 rpm by the flame photometer method. Sodium and potassium concentrations were determined also in the 2 hour urine samples collected. Afterwards, the separate 2-hour samples of urine were mixed and the total quantity of urine excreted over the period of sodium administration was analyzed for sodium and potassium. Glomerular filtration and renal plasma flow were calculated during the control period and at the termination of the oral sodium loading Total quantity of sodium and potassium excreted (UV) during the same periods, as well as during the 2-hour collection periods, was calculated. In addition, percentage of filtered sodium excreted was calculated during all clearance periods

Plasma volume study Eleven healthy Peruvian males between the ages of 20 and 28 years (including seven of the former subjects who volunteered for this test one month after completion of the previous one) were studied. All of the same conditions were maintained as before, except for the fact that no renal clearances were performed. Instead, at 9 A M on the day of the beginning of the test, plasma volume was measured by the four sample techniques using T-1824 (11) Again the subjects received a quantity of the saline solution equivalent to 10 per cent of body weight via gastric tube uniformly by constant drip over a 21-hour period. After completion of administration of the saline solution (8 A M), plasma volume determination was repeated as on the day before.

RESULTS

Hemodynamics

In Table I are listed glomerular filtration rate, renal plasma flow and plasma volume during the control period and during the period just after the administration of a quantity of the isotonic saline solution equivalent to 10 per cent of body weight In eleven of the twelve subjects the glomerular filtration rate increased significantly over the control value and in only one individual did it stay the same The average value shows a significant increase (+ 32 per cent) over the control value after the sodium load On the other hand, renal plasma flow increased over the control value in six of the twelve subjects, decreased in five and stayed the same in one The average value of all the subjects does not show a significant increase over the control value The plasma volume increased over the control value in four of twelve individuals, decreased in six and remained the same in one after the administration of the saline solution The average value of all the subjects shows no significant change over the control value Venous pressure was measured during the control period (average, 104 mm) and after sodium load-

TABLE I

Hemodynamic studies in normal subjects with oral sodium loading

Subject	No of clearances	Glomerular filtration cc./min./1.73 m²			Renal plasma flow cc /min /1.73 m²			Plasma volume cc		
		Control*	After*	Diff	Control*	After*	Diff	Control	After	Diff
J C	3	105	161	55	614	725	111	3,055	3,358	303
M C	2	160	253	93	599	925	326			
L M	2	129	195	66	752	730	−22	2,110	2,400	290
P L	2	162	198	36	1,099	1,375	276			
E R	3	130	126	−4	648	577	−71	2,610	2,483	−127
A R	3	108	139	31	759	587	−172	3,354	3,600	246
A V	3	138	167	29	668	668	0			
J G	3	103	146	43	635	605	−30			
V F	3	118	163	45	631	750	119			
L Q	3	145	186	41	825	863	38	3,027	2,731	−296
R C	3	102	133	31	685	649	−36	3,035	3,048	13
C G	3	114	137	23	728	768	40	2,800	2,650	−150
A L								3,510	3,097	−413
J R								2,256	2,320	64
J E								2,720	2,455	−265
J M								2,941	2,463	−478
Average values		126	167	41†	720	768	48	2,856	2,782	− 74
Standard error of average				6 9			40 7			84

* Each number represents the average value of the clearance periods
† Significant at the 1 per cent level

TABLE II

Water and electrolyte excretion in normal subjects with oral sodium loading

Subject	No of clearance periods	Urine volume* cc/min		U_NV* mEq./min			U_X* mEq./L.		U_KV* mEq./min.		U_K* mEq./L.	
		Control	After	Control	After	Sum	Control	After	Control	After	Control	After
J C	3	2 65	4 22	0 345	0 660	1 005	135.3	158 8	0 0689	0 0390	26 4	9 3
L M	2	3 79	3 03	0 310	0 520	0 830	85 7	172 0	0 0425	0 0449	11 7	14 8
P L	2	4 88	4 40	0 560	0 934	1 494	118 8	212 5	0 0913	0 0892	19 8	20 3
M C	2	9 75	3 85	0 526	0 718	1 244	54 1	187 0	0 0911	0 0294	9 4	7 6
E R	3	3 80	6 53	0 485	0 701	1 186	130 6	113 2	0 1168	0 0684	31 2	10 8
A R	3	4 02	3 65	0 528	0 572	1 100	148 4	155 5	0 0473	0 0277	13 1	7 6
A V	3	3 96	4 15	0 403	0 794	1 197	103 0	192 9	0 1021	0 0543	26 2	13 2
J G	3	4 69	1 63	0.286	0 396	0 682	64 4	242 0	0 0642	0 0437	14 1	26 7
V F	3	4 14	4 43	0 457	0 758	1 215	125 6	1,6 3	0 0586	0 0,01	16 4	16 5
L. Q	3	1 66	4 01	0 422	0 751	1 173	265 0	91 6	0 0367	0 0260	23 0	4 9
R. C	3	1 36	5 04	0.345	0 841	1 186	49 4	167 1	0 0390	0 0400	4 9	8 0
C G	3	5 29	9.33	0 613	0 961	1.574	124 7	103 2	—	—	—	—
Average value		4 16	4.52	0 440	0 717		117 1	164 3	0 0690	0 0484	17 8	12 7

Analysis of variance of $U_N V$ †

Source	df	Sum of squares	Mean square	F
Mean	1	8 0342		
Total	23	0 8804		
Control vs After	1	0 4611	0 4611	68 82 ‡
Subjects	11	0 3455		
Interaction	11	0 0738	0 0067	
				$F_{.01}$ 9 65

* Each number represents the average value of the clearance periods.
† $U_N V$ = Total Na excretion U_X = Na concentration in urine excreted
$U_K V$ = Total K excretion U_K = K concentration in urine excreted
df = Degrees of freedom
‡ Significant at the 1 per cent level

ing (average 133 mm) in nine individuals but no significant change was noted.

Excretion of water and electrolytes

In Table II are shown the concentrations and total quantity of the ions Na and K excreted during the clearance periods performed before and after the administration of the saline solution The concentrations of sodium increased significantly after sodium loading while the concentration of potassium decreased The total quantity of sodium excreted augmented significantly after sodium loading (+ 63 per cent) while potassium diminished (− 30 per cent) The sodium excreted during the control period represented 2 58 per cent of the filtered load while the sodium excreted after the administration of the sodium load represented 3 33 per cent of the filtered load Although the volume of urine increased in seven of twelve individuals the average urine volume after sodium loading showed no significant increase over the

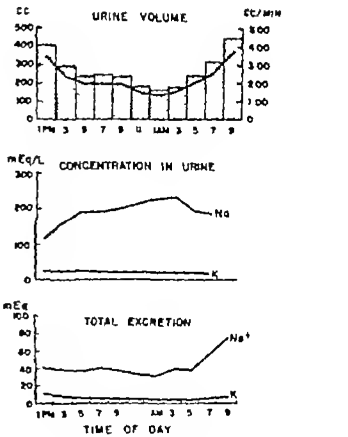

FIG. 1 DIURNAL VARIATIONS WITH ORAL SODIUM LOADING

TABLE III

*Fluid and electrolyte balance in normal subjects with oral sodium loading**

Subject	Fluid intake cc	Electrolyte intake mEq			Urinary output cc	Urinary excretion mEq		Weight change Kg	
		Na	K	Cl		Na	K	After Na load	3 hrs after Na load
M C	6,650	822	0	558	2,620	432	40	+2.1	+1.6
L M	5,300	685	0	465	3,340	455	43	+1.5	+0.7
	5,300	726	0	493	4,000	480	46	+0.5	
P L	6,000	822	0	558	4,860	690	53	+0.3	−1.1
E R	5,000	685	0	465	3,020	382	38	+1.7	+0.9
	5,400	740	0	502	2,540	315	62	+1.7	
A R	6,000	810	0	534	2,925	375	22	+2.4	+1.4
	6,000	810	0	534	960	232	38	+4.4	
A A	6,600	904	0	614	4,960	660	66	0.0	−1.1
J G	7,000	959	0	651	2,000	540	68	+3.3	+2.9
A F	6,000	822	0	558	3,600	490	59	+0.7	−0.5
I Q	6,000	822	0	558	3,500	517	39	+1.5	
	6,000	822	0	558	2,680	493	66	+2.2	
R C	7,000	959	0	651	3,300	609	64	+3.3	+2.5
	7,000	959	0	651	3,060	536	59	+3.3	+2.1
C G	6,300	863	0	586	3,900	554	78	+1.1	−0.4
	6,000	822	0	558	2,780	378	98	+2.0	
J R	5,000	685	0	465	1,540	408	55	+1.8	
J F	5,300	726	0	493	4,540	654	91	+0.3	
J M	6,300	863	0	586	1,540	442	31	+2.9	
Average	6,000	815	0	552	3,083	472	56	+1.85	+0.82

* Table does not include one subject in which the sodium load produced diarrhea and another who had large saliva loss.

control value due to the fact that the control value itself represented a slight water diuresis.

In Figure 1 are demonstrated the average urine volume, concentration of sodium and potassium and total excretion of sodium and potassium of the 2-hour urine collections during the administration of the saline solution. The first 2-hour sample showed an increase of water and sodium excretion as compared to the values prior to the administration of the saline solution. Water, sodium and potassium excretion all decreased during the night hours with sodium excretion rising notably during the early morning hours above the values encountered at the beginning of the test on the previous day. Sodium concentration in the urine rose during the administration of the sodium load with only a moderate effect noticeable due to changes in water excretion, while potassium concentrations remained relatively constant regardless of water excretion.

In Table III are listed the total intake and total output of water and electrolytes during the administration of the sodium load, together with the changes of weight affected by the sodium administration. It is noted that 51 per cent of the water and 58 per cent of the sodium administered were excreted via the kidney during the period of oral administration of sodium. From the weight data, it can be observed that at the completion of the sodium loading only 31 per cent of the water administered (1.85 Kg.) was retained, which rapidly diminished to 14 per cent (0.82 Kg.) three hours after the termination of the sodium administration. The different responses of a single subject receiving the same sodium load on two separate occasions are also given.

Blood analyses

Table IV demonstrates the changes in hematocrit, total plasma proteins, plasma concentration of sodium, potassium, chloride and bicarbonate and erythrocyte concentration of sodium and potassium before and immediately after the administration of the sodium load. Although some of

TABLE IV

Blood analyses in normal subjects with oral sodium loading

Analyses	Control Average ± S.D	After Na loading Average ± S D
Hematocrit %	46.2 ± 2.48	44.0 ± 7.2
Tot. Proteins *gm* %	7.66 ± 0.34	7.17 ± 0.47
Plasma Na *mEq*/L	139.1 ± 3.51	137.2 ± 3.39
Plasma K *mEq*/L	4.13 ± 0.37	3.86 ± 0.30*
Plasma Cl, *mEq*/L	101.3 ± 0.95	100.7 ± 0.55
Plasma HCO_3 *mEq*/L	24.6 ± 0.41	23.0 ± 0.51*
RBC K *mEq*/L	90.8 ± 3.5	87.9 ± 4.2*
RBC Na *mEq*/L	14.5 ± 0.35	14.7 ± 1.84

* The difference between the control value and the value after the sodium load has statistical significance

these values changed significantly due to the administration of the large quantities of saline solution, none of the changes appear to be of any real importance.

DISCUSSION

The excretion of 58 per cent of a large oral sodium load during the period of its administration challenges the widely held belief that man, unlike the dog, is sluggish in excreting sodium (2). Since previous studies in the literature concerned with sodium loading in man have all been performed giving smaller total amounts of physiologic sodium chloride solution (1 to 3 liters) but by more rapid intravenous infusion (13 to 65 cc per min), it is possible that the previously unreported experimental conditions used in this study are responsible for the observed difference.

Earlier investigators have held that man excreted sodium sluggishly compared to the dog due to the fact that glomerular filtration did not increase in man with the intravenous administration of sodium chloride. In the present study, however, there was usually a consistent and significant increase of glomerular filtration at the termination of oral sodium loading. Since the percentage of filtered sodium excreted increased significantly from 2.58 per cent to 3.33 per cent, it is evident that decreased tubular reabsorption over and above the increased amount filtered by the glomerulus accounted for a part of the augmented sodium excretion. Even though it is difficult to compare the results of this study with prior ones, it can be stated, nevertheless, that water and sodium excretion after oral sodium loading fell to values between those reported in non prehydrated (2) and prehydrated (12) subjects maintained in the recumbent position.

The magnitude of the sodium and water diuresis after oral sodium loading is masked somewhat by the control values, since these latter values themselves demonstrated a slight water and sodium diuresis compared to data in the literature. The slight water diuresis during the control period was due to the administration of a small quantity of water before the commencement of the test. The slight sodium diuresis is more difficult to explain but is most likely due to an augmented ingestion of sodium in the daily Peruvian diet prior to the test.

Although no attempt was made to study renal hemodynamics during the course of the administration of the sodium load, the gross data of sodium and water excretion on the 2 hour samples collected during that time demonstrated the well known diurnal variations of water and sodium excretion without sodium loading, but at higher levels than reported under normal circumstances (13).

SUMMARY

1. Sixteen healthy young Peruvian males were given a quantity of an isotonic saline solution equivalent to 10 per cent of body weight in the recumbent position by constant drip via a gastric tube over a period of 21 hours. Renal hemodynamics and electrolyte excretion were studied in twelve of the subjects and plasma volumes in eleven of them before and after the administration of the sodium load.

2. Glomerular filtration rate increased an average of 32 per cent over the control value after administration of the sodium load, while effective renal plasma flow did not change significantly.

3. While body weight was increased significantly at the termination of the sodium load, plasma volume did not change significantly. The increase in body weight largely disappeared within 3 hours after termination of the load.

4. During the period of administration of the sodium load, 58 per cent of the administered sodium and 51 per cent of the administered water were excreted via the kidneys. The concentration and total quantity of sodium excreted in the urine increased significantly at the termination of the sodium load. An increased filtration of sodium by the glomeruli as well as a decreased reabsorp

tion of sodium by the tubules are considered to be responsible for this phenomenon. Water excretion was augmented also but its significance was masked by the slight water diuresis present in the control values. The diurnal variations of sodium and water excretion during the course of administration of the sodium load were evident, but at higher levels than under normal circumstances.

5. Total excretion of potassium as well as potassium concentration in the urine decreased after sodium loading.

6. There were no important changes in plasma concentrations of red blood cells, total proteins or electrolytes.

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OBJECTIVE EVALUATION OF PATIENTS WITH RHEUMATIC DISEASES II. PAPER ELECTROPHORETIC STUDIES OF SERUM GLYCOPROTEIN AND PROTEIN FROM PATIENTS WITH RHEUMATOID ARTHRITIS[1]

By GEORGE STIDWORTHY, R. W. PAYNE, CLARA L. SHETLAR, AND M. R. SHETLAR

(From the Research Laboratory, V. A. Hospital, and the Departments of Biochemistry and Pharmacology, University of Oklahoma School of Medicine, Oklahoma City, Okla.)

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Serum glycoprotein concentrations serve as a valuable index of the intensity of inflammatory activity in the rheumatic diseases (1), particularly when the glycoprotein level is considered in relation to the serum protein with which it is bound, *i.e.*, as the polysaccharide protein ratio (PR). The typical changes of serum protein (lowering of albumin, increase of globulin) in rheumatoid arthritis are well known (2).

The development of techniques for serum protein and glycoprotein analyses by filter paper electrophoresis permits a detailed study of the changes in each of the serum protein components as well as the distribution of carbohydrate bound to these components (3). The relationship of these various components to the severity of inflammatory activity of rheumatoid arthritis has consequently been investigated.

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METHODS

Laboratory. Paper strip electrophoresis was performed essentially as described by Block, Durrum and Zweig (4) using Durrum type cells (Spinco Model R). Electrophoretic runs were made at room temperature (78° to 85°) for 16 hours on Whatman 3MM filter paper strips saturated with 0.075 M Barbital buffer (pH 8.6) using a constant current of 5 milliamps for each cell of 8 strips. A volume of 10 microliters was applied to the paper for protein fractionations and 30 microliters for glycoprotein separations. All samples were run in duplicate. Protein was visualized on the paper strips by staining with bromophenol blue-zinc sulfate as described by Block, Durrum, and Zweig (4). The glycoprotein color development on the paper strips was accomplished by the Periodic-acid Schiff reaction largely as described by Koiw and Gronwall (5). However, the reducing solution was prepared as described by Roboz, Hess and Forster (6).

Quantitation of the developed strips was made with a servo type recording photometer and automatic integrator (Spinco Model R Analytrol). Protein strips were scanned through blue filters, glycoprotein strips through green filters (Klett 52). The total area under the protein curve was equated to the total serum protein as determined by the biuret reaction of Weichselbaum and Shapiro (7) and the glycoprotein curve to the total serum glycoprotein as determined by the tryptophan

TABLE 1

Serum protein fractions as estimated by paper electrophoresis

Group	No.	Protein as per cent of total protein of: Albumin		α_1	β	γ
Normal	13	57.3 ±1.7‡	4.9 ±0.3	9.4 ±0.	11.9 ±0.6	16.3 ±1.3
Rheumatoid arthritis						
Clinical activity 1	8	49.5 ±0.8	5.3 ±0.4	10.6 ±0.6	13.7 ±0.1	20.9 ±1.4
Clinical activity 2	13	44.9 ±1.2	6.4* ±0.4	11.1 ±0.4	14.3 ±0.6	23.3* ±1.2
Clinical activity 3 and 4†	12	39.5* ±2.0	6.8 ±0.3	14.7 ±0.5	15.4 ±0.6	23.7 ±2.3

* Significantly different from the normal group at the 1% level of probability.
† Composed of eight with activity 3 and four with activity 4.
‡ Figures following the ± sign are standard errors of the mean of each group.

TABLE II

Protein bound polysaccharide† of serum fractions as estimated by paper electrophoresis

	No	Mg % of polysaccharide of						
		Total	Albumin	α_1	α_2	β	γ	Seromucoid
Normal	15	115 ±4.1	14.8±0.7	16.3 ±0.9	33.6 ±2.3	28.3 ±1.5	22.5±1.3	11.0 ±1.4
Rh arthritis								
Clin activity 1	8	150*±3.6	14.7±1.1	19.7*±1.3	51.9*±2.5	35.9*±1.6	27.8±1.8	20.5*±1.6
Clin activity 2	13	176*±14.6	14.3±1.8	28.0*±1.7	66.6*±2.9	36.7*±1.4	29.0±2.5	30.5*±1.7
Clin activity 3 and 4‡	12	197*±5.9	14.2±0.9	34.5*±3.0	81.1*±3.9	37.6*±3.2	29.1±2.2	30.3*±0.8

	No	Polysaccharide as per cent of the protein (PR) of					
		Total protein	Albumin	α_1	α_2	β	γ
Normal	15	1.48 ±0.03	0.33 ±0.02	4.27 ±0.28	4.67 ±0.30	3.04±0.21	1.81±0.09
Rh arthritis							
Clin activity 1	8	1.91*±0.03	0.37 ±0.05	4.84*±0.11	6.29*±0.35	3.47±0.31	1.79±0.21
Clin activity 2	13	2.26*±0.06	0.41 ±0.05	5.89*±0.16	7.87*±0.44	3.42±0.16	1.61±0.13
Clin activity 3 and 4‡	12	2.73*±0.06	0.52*±0.04	6.96*±0.40	7.93*±0.32	3.32±0.26	1.77±0.13

* Significantly different from the normal group at the 1 per cent level of probability

† Bound hexose as determined by the tryptophan method (8). Hexosamine component of the polysaccharide complex is not included

‡ Composed of eight with activity 3 and four with activity 4

method of Shetlar, Foster and Everett (8). The quantitations of individual fractions were made by calculating the percentage of the total area contributed by the areas representing each of the various fractions. The areas representing each of the glycoprotein fractions were selected by comparing the glycoprotein densitometer curve with the corresponding protein curve.

Seromucoid was determined by the method of Weimer and Moshin (9).

Clinical. An estimate of clinical activity of the rheumatoid process was assigned at each clinic visit as described in a previous report (10). Current disease activity was thus grossly appraised as

Activity 1—No inflammatory activity
Activity 2—Mild activity
Activity 3—Moderate activity
Activity 4—Severe activity

RESULTS

Results of the analyses of the serum protein and glycoprotein fractions from patients with rheumatoid arthritis of various degrees of severity are summarized in Tables I and II. Typical electrophoretic strips of a normal serum are depicted in Figure 1 and those of a patient with severe rheumatoid arthritis in Figure 2.

With increasing severity of the rheumatoid process an increase occurred in all the globulin fractions, being most marked in the α_2 globulin. A corresponding significant decrease in the serum albumin fractions occurred; indeed it would appear that the decreasing serum albumin is a slightly more sensitive index of activity than the serum globulin increase.

The most striking changes in serum glycoprotein of patients with rheumatoid arthritis occurred in those carbohydrate moieties associated with the α_1 and α_2 protein fractions. This increase is in excess of the protein increase in the α_1 and α_2 fractions resulting in an increased percentage of bound carbohydrate in these fractions (Table II). On the other hand the increases in the β and γ fractions merely parallel the changes in protein contents of these respective fractions.

Correlation coefficients of protein, seromucoid and glycoprotein fractions with clinical activity of the rheumatoid arthritis patients are presented in Table III. Clinical activity was found to have a significant positive correlation with total PR, α_1 and α_2 glycoprotein, seromucoid and α_2 globulin protein, and a negative correlation with serum albumin. Seromucoid exhibited significant correlation with total glycoprotein (expressed either as mg per cent or as PR) and with α_1 and α_2 glycoprotein. The total PR was correlated with the PR's of the α_1 and α_2 globulins. Total glycoprotein (in mg per 100 ml.) exhibited a low but sig-

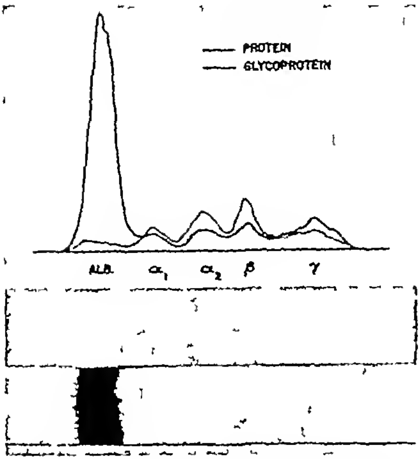

FIG. 1 TYPICAL PAPER STRIP ELECTROPHORETIC PATTERNS OF NORMAL SERUM WITH CORRESPONDING DENSITOMETER TRACINGS

The solid line indicates protein concentration the dotted line, glycoprotein concentration. The top strip is stained with Periodic acid Schiff *reagents for glycoprotein* and the bottom with bromophenol blue for protein.

The total hexose glycoprotein of this serum was 108 mg per 100 ml distributed among the fractions as follows Albumin, 13.2 mg per 100 ml α_1 14.5 α_2 27.6 β 30.2 and γ 22.4 The total protein of this sample was 7.42 grams per 100 ml

nificant correlation with α_1 glycoprotein but not with α_2 glycoprotein

DISCUSSION

Changes in serum protein produced by various types of rheumatic diseases have been reported The data of Table I are confirmatory of earlier work in which a decrease of serum albumin and an elevation of the globulin fractions was noted The observation that serum albumin as determined by a 26 per cent sodium sulfate salting out procedure has a negative correlation with activity of rheumatoid arthritis (11) is further confirmed by paper electrophoresis studies (Table III) The correlation coefficient of − 0.526 for the salting out method is very close to the − 0.563 found in the present study Use of the electrophoretic method allows further investigation of relationships between clinical activity and protein fractions The finding of a positive correlation of 0.635 for α_2 globulin with clinical activity is of interest as elevations of this fraction have been noted in many inflammatory conditions No other protein fraction was significantly correlated with activity It may be implied that albumin and α globulin fractions are more rapidly affected than are other fractions by inflammatory activity in rheumatoid arthritis insofar as their protein moieties are concerned

The concentration of serum glycoprotein in patients with rheumatoid arthritis serves as an index of the inflammatory activity of this disease. As can be seen in Table II the most striking elevation of serum glycoprotein in patients with rheumatoid arthritis is found in carbohydrate bound

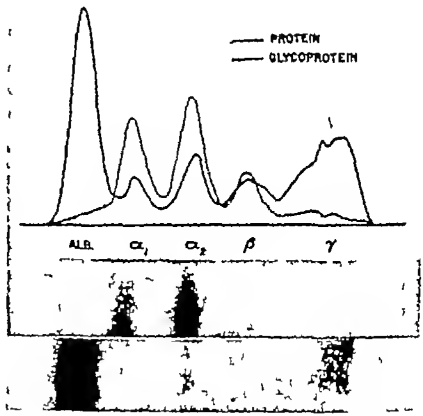

FIG 2. TYPICAL PAPER STRIP ELECTROPHORETIC PATTERNS AND CORRESPONDING DENSITOMETER TRACINGS OF THE SERUM OF A PATIENT WITH ACTIVE RHEUMATOID *ARTHRITIS*

The solid line indicates protein concentration the dotted line, glycoprotein concentration. The top strip is stained with Periodic acid Schiff reagents for glycoprotein and the bottom *strip* with bromophenol blue for protein.

Total hexose glycoprotein of this serum was 208 mg per 100 ml. distributed among the *fractions* as follows Albumin, 9 6 mg per 100 ml. α_1 63 4 α_2 79.7 β 33.7 and γ 21 6. Total protein of this sample was 7.22 grams per 100 ml

TABLE III

Summary of correlation coefficients

	r*	t value†	P
Correlation with clinical activity			
Total PR‡	0.914	12.50	.01
α_1 PR‡	0.559	3.75	.01
α_2 PR‡	0.296	1.72	.10
Albumin % of total protein	−0.563	3.79	.01
α_1 % of total protein	0.110	0.64	.30
α_2 % of total protein	0.635	4.58	.01
β % of total protein	0.183	1.34	.15
γ % of total protein	0.306	1.79	.10
α_1 polysaccharide, mg %	0.654	4.82	.01
α_2 polysaccharide, mg %	0.684	5.21	.01
Seromucoid	0.689	3.49	.01
Correlation with seromucoid			
Total PR‡	0.786	5.09	.01
Total polysaccharide, mg %	0.826	3.85	.01
Alb. polysaccharide, mg %	−0.224	0.92	.25
α_1 polysaccharide, mg %	0.826	5.88	.01
α_2 polysaccharide, mg %	0.603	3.02	.01
β polysaccharide, mg %	0.448	1.69	.15
γ polysaccharide, mg %	−0.082	0.21	.45
Correlation with total polysaccharide			
α_1	0.459	2.88	.01
α_2	0.114	0.64	.35
Correlation with total polysaccharide-protein ratio			
α_1 PR‡	0.686	5.24	.01
α_2 PR‡	0.706	5.55	.01

* r = Correlation coefficient

† $t = \frac{n^{\frac{1}{2}} r}{(1 - r^2)^{\frac{1}{2}}}$ $n = N - 2$

‡ Polysaccharide (bound hexose) as per cent of the protein involved

to the α-globulins. This increase is in excess of α globulin protein increases since there is a disproportionately large percentage of glycoproteins bound to both α_1 and α_2 globulins in the active phases of the disease studied. Some of this elevation is probably due to increases in the seromucoids which have electrophoretic mobilities at pH 8.6 similar to the α-globulins. Furthermore the high correlation coefficients of α_1 globulin carbohydrate (0.826) and α_2 globulin carbohydrate (0.603) with seromucoid suggest a close relationship of these components in rheumatoid arthritis. However the seromucoid levels (Table II) are not high enough to account for all of the carbohydrate increases found in the α_1 and α_2 fractions. One may well speculate that carbohydrate rich fractions with mobilities at pH 8.6 similar to α_1 or α_2 globulin as yet not definitely characterized are elevated in the sera of patients with rheumatoid arthritis.

The carbohydrate bound to albumin was found to be relatively low by the procedure followed in this study. Only slight changes of albumin carbohydrate percentage occurred in patients with rheumatoid arthritis. This is in contrast to data obtained by salt fractionation methods as previously reported (11) in which striking increases of albumin bound carbohydrate were found in active rheumatoid arthritis. The most likely explanation of the disparity between the data obtained by the two methods is that the albumin fraction obtained by salt fractionation contains appreciable amounts of carbohydrate rich globulin and thus has a higher carbohydrate content.

The significant changes in serum α_2 globulin, the bound carbohydrate of α_1 and α_2 globulin and of seromucoid with increasing clinical activity of rheumatoid arthritis patients are of considerable interest. The technique of filter paper electrophoresis would appear to have advantages in that only minute amounts of serum are required for analysis and the entire spectrum of protein and glycoprotein may be investigated. However, data obtained from total serum polysaccharide-protein ratio as obtained by chemical methods, which are a necessary preliminary to paper strip quantitation, are apparently as satisfactory as any of the fractionation data for use in evaluating activity of the rheumatoid process.

SUMMARY

Paper electrophoretic techniques have been applied to analyses of serum protein and glycoprotein components from patients with rheumatoid arthritis of various degrees of severity. The increase of serum glycoprotein in patients with rheumatoid arthritis is due to 1) increases of the carbohydrate rich globulin fractions relative to the carbohydrate poor albumin fraction and 2) an increase of the carbohydrate content of the α_1 and α_2 globulin fractions.

Changes in the α globulins are apparently most closely related to inflammatory activity in rheumatoid arthritis as the carbohydrates associated with α_1 and α_2 globulin and the α_2 globulin protein increased with increasing clinical activity. A decrease of albumin protein occurred with increasing severity of the disease.

Total serum glycoprotein (expressed as hexose polysaccharide as a percentage of the serum protein) exhibited the highest correlation with clinical activity, and consequently is to be recommended as the most satisfactory laboratory method of those studied for evaluation of the status of patients with rheumatoid arthritis

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THE ABSORPTION OF RADIOIRON LABELED FOODS AND IRON SALTS IN NORMAL AND IRON-DEFICIENT SUBJECTS AND IN IDIOPATHIC HEMOCHROMATOSIS

By ROBERT B CHODOS,[1] JOSEPH F ROSS,[2] LEONARD APT,[3] MYRON POLLYCOVE,[4] AND JAMES A. E. HALKETT

(From the Radioisotope Unit and Medical Service, Veterans Administration Hospital, Boston, the Department of Medicine, Boston University School of Medicine, the Department of Pediatrics, Harvard Medical School, and the Department of Medicine, Tufts University School of Medicine, Boston, Mass)

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It is generally accepted that, in the absence of bleeding or pregnancy, approximately 1 mg of iron is lost per day from the body (1–11) As a corollary, the quantity of iron in the body is largely determined by the amount of iron absorbed from the gastrointestinal tract Fundamental knowledge of iron absorption has been obtained by balance studies (2, 4, 12–18), by determination of the increase in the serum iron level (19) or circulating hemoglobin (20–21) after oral iron administration, and by evaluation of the percentage of orally administered radioiron incorporated into hemoglobin (22–26) or accounted for in both hemoglobin and feces (7, 27)

The present investigation was undertaken to compare the absorption of egg and vegetable iron with that of iron salts fed to normal subjects, to patients with iron-deficiency, and to patients with idiopathic hemochromatosis The method of study used was similar to that introduced by Dubach, Callender, and Moore (27) These investigators showed that the quantity of iron used in hemoglobin formation may not always be a true index of iron absorption (27, 28) Since a negligible quantity of iron is excreted into the intestinal tract (1–3, 7–8 11) and since stool iron is almost entirely unabsorbed dietary iron (2), additional information concerning iron absorption may be obtained by determining both the percentage of orally administered radioiron incorporated into hemoglobin and the percentage recovered in the feces The percentage of the oral dose not recovered in hemoglobin and feces may be considered an approximate measure of the quantity of iron deposited in tissue stores In certain patients this figure may be subject to a significant experimental error due particularly to incomplete stool collection

EXPERIMENTAL SUBJECTS AND METHODS

Three groups of patients were studied

1 *Normal subjects* This group consisted of 32 male patients with uncomplicated dermatitis, psychosomatic or psychiatric illnesses, or neurologic diseases All had normal hematologic and serum iron values Iron stores were not specifically measured by tissue biopsy (bone marrow or liver) Blood loss was excluded by history and stool examination Systemic diseases that might influence iron absorption, utilization, or the level of iron stores were excluded by appropriate laboratory and clinical evaluation

2 *Subjects with deficient iron stores* This group comprised 15 patients with evidence of chronic blood loss The red blood cells were hypochromic and microcytic, serum iron levels were less than 50 micrograms per 100 ml. and a definite history of blood loss was obtained in each case.

3 *Subjects with excess iron stores* This group of 9 patients had idiopathic hemochromatosis The diagnosis was established by clinical features, elevated serum iron level, and by liver or skin biopsy

The number of absorption studies and the form in which the iron was administered to these subjects are summarized in Table I

Red blood cell indices were determined for each subject using equipment certified by the Bureau of Standards Serum iron was measured by the method of Kitzes, Elvehjem and Schuette (29) The whole blood, red cell and plasma volumes were estimated using a radiophosphorus tagged red cell method (30)

Following these studies, tracer doses of radioiron were given by mouth to the fasting subject either as ferrous or

[1] Present address VA Hospital Syracuse, New York.

[2] Present address School of Medicine, University of California Los Angeles California.

[3] Present address Wills Eye Hospital, Philadelphia, Pennsylvania.

[4] Present address Donner Laboratory University of California, Berkeley California.

TABLE I

Summary of iron absorption studies

Group	No	Iron 59 administered: As $FeCl_2$	As $FeCl_3$	As labeled food: Eggs	Vegetables	Total no. of studies
Normal	32	25	2	14	6	47
Iron deficient	15	14	2	3	2	21
Hemochromatosis	9	13	0	1	2	16
Total	56	52	4	18	10	84

ferric chloride or as a radioiron labeled food. Ferrous chloride was prepared by the reduction of ferric chloride with powdered ascorbic acid or in 2 studies with cysteine. The oral dose of Fe^{59} [a] used in the iron salt absorption studies ranged from 12 to 50 microcuries and from 7 to 23 microcuries in the food iron absorption studies.

In all but 7 studies evaluating the absorption of ferrous chloride, ascorbic acid tablets (total of 0.5 to 1.0 gm.) were administered concomitantly with the radioactive iron salt. A variable quantity of non-radioactive carrier iron ranging from 4 or 5 mg. to 80 mg. was usually added to the tracer dose. The smaller carrier doses were used if the absorption of food iron had been previously studied in the subject, since this dose range approximated the quantity of iron administered in the labeled foods. Larger carrier doses were used in the remaining iron salt absorption studies.

The Fe^{59} labeled foods were chicken eggs and vegetables. The techniques developed for preparation of these labeled foods will be reported separately (31). The quantity of iron in a given oral dose of labeled food was determined by the method of Kitzes, Elvehjem, and Schuette (29) on an aliquot of the food substance after Kjeldahl digestion with concentrated nitric, sulfuric, and perchloric acid. Representative iron content of the various foods used is tabulated in Table II.

Radioiron labeled foods were administered after a night's fast. Two pieces of bread (containing approximately 1 mg. of iron) without butter and black coffee (no iron) with sugar were eaten with the labeled food. Eggs were served scrambled and vegetables were boiled

TABLE II

Iron content of labeled food products

Food product	No. of assays	Iron content (mg./100 gm.): Range	Average	Iron administered (mg.): Range	Average
Chicken eggs	16	2.8–4.7	3.8	3.9–10.0	6.0
Swiss chard	5	0.6–0.9	0.8	2.2–4.4	2.9
Beet greens	5	1.1–2.5	1.8	2.0–4.8	3.0

[a] The radioactive iron used in these studies was prepared in the atomic pile at Oak Ridge. The specific activity ranged from 455 to 4,237 mc. per gm. Fe.

and served with the cooking liquid. Eight ounces of orange juice were given with scrambled eggs in a single study.

After iron administration, stools were collected until less than 1 per cent of the oral tracer dose was recovered in a 24-hour collection. Samples of blood were obtained at 2 to 5-day intervals. In most instances, blood samples were obtained until a plateau of constant activity was reached.

The preparation of the collected materials for counting was relatively simple. Water was added to feces in the large collecting bottles, total weight determined, and the mixture homogenized by an Osterizer or an Equipoise shaker. After mixing three aliquots by weight were transferred to screw cap bottles holding 25 ml. of material. Since the quantity of radioiron present in plasma after the first 24 hours was not significant, blood samples were processed for assay by pipetting 25 ml. of whole blood into similar bottles. Appropriate standards representing the administered oral dose of radioactive iron salt or food iron were similarly prepared. The standard for iron salts consisted of a portion of the solution taken per mouth by the subjects while that for labeled foods

TABLE III

Recovery of radioiron added to feces

Experiment	No. of stool samples ctd.	Net cps total stool (ave.)	Net cps std. (ave.)	% Recovery
1	8	640.63	664.4	96.42
2	8	644.74	664.0	97.09
3	9	668.96	662.6	100.95
4	9	627.90	652.3	96.25

was made up from weighed aliquots of scrambled eggs or cooked vegetables. These were digested in concentrated nitric acid and diluted to a 25-ml. volume in vials like those used for counting the stool and blood samples.

Gamma radiation of the prepared samples was quantitatively measured by Geiger-Mueller counting tubes. Initially a Sylvania GG306 all metal, bismuth cathode tube was used. This heavily shielded tube was mounted in a horizontal position in a plastic frame and the sample bottles were placed as close as possible beneath the tube, with the long axis of the sample bottle parallel to the tube. Counts were recorded on a Tracerlab Autoscaler. The counting efficiency of this arrangement for Fe^{59} was 0.4 per cent and 180 cps represented 1 microcurie. Statistical analysis of the whole sampling positioning and counting technique revealed a potential error that did not exceed ± 5 per cent.

Later another type of Geiger-Mueller tube was used, the Texas Well Counter.[b] This tube was approximately 8 to 10 times as sensitive as the GG306. All food absorption studies utilized the Texas Well Counter, counts being recorded by a Berkeley Decimal Scaler.

[b] Welch Allyn Company, Skaneateles, New York.

7) was a female who had had a subtotal gastric resection three years previously for a bleeding ulcer Subsequent to this operative procedure she developed a profound iron deficiency anemia in the absence of further bleeding other than that associated with her menses This anemia was reported to be refractory to therapy with ferrous sulfate When she received labeled eggs and chard in two separate studies she was able to absorb and incorporate into hemoglobin only 0 6 and 3 per cent, respectively Virtually all the remaining Fe^{59} was recovered in the stools so that less than 0 1 mg of iron was actually absorbed In striking contrast, when a comparable quantity of Fe^{59} was administered as ferrous chloride (4 0 mg of iron), 88 per cent was absorbed and incorporated into hemoglobin, representing the absorption of 3 5 mg of iron When the carrier dose of ferrous chloride was increased to approximate the 80 mg of iron received daily on an average therapeutic regimen of ferrous sulfate, 32 per cent of the dose was absorbed and incorporated into hemoglobin, representing the absorption of 26 mg of iron

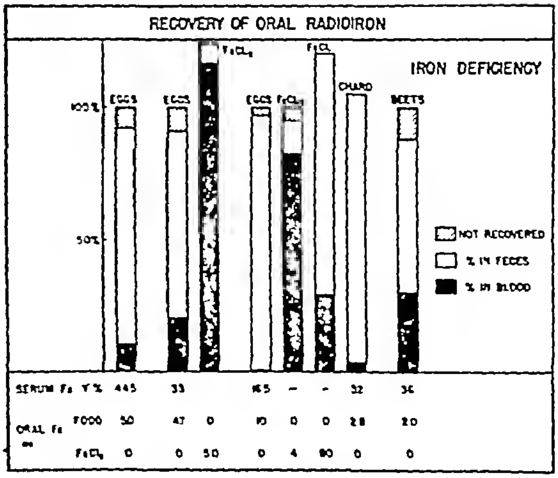

FIG 6 COMPARATIVE RECOVERY OF RADIOIRON BY IRON-DEFICIENT PATIENTS FOLLOWING THE ADMINISTRATION OF EITHER LABELED FOOD (EGGS, VEGETABLES) OR FERROUS CHLORIDE

Studies 2 and 3 were carried out in sequence on one patient and studies 4 to 7 inclusive on a second patient.

Hematologic values of patients

	Range	Mean
Hematocrit (%)	29 0–40 0	32 0
Hemoglobin (gm %)	6 0–11 9	7 5
MCHC (%)	19 0–30 0	23 0

In summary, iron as ferrous chloride is absorbed in larger quantity by iron-deficient subjects than is the iron in eggs and vegetables However, food iron is absorbed and used to a much greater extent by patients with iron deficiency than by normal subjects

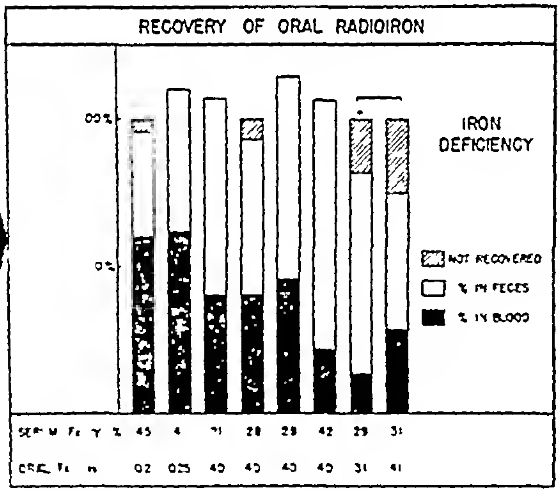

FIG 5 RECOVERY OF RADIOIRON BY PATIENTS WITH DEFICIENT IRON STORES FOLLOWING THE ADMINISTRATION OF AN IRON SALT

The labeled salt was administered as ferric chloride (+++ above column) or as ferrous chloride (no symbol above column) with 31 to 41 mg of carrier iron No carrier iron was administered in studies 1 and 2

Hematologic values of patients

	Range	Mean
Hematocrit (%)	21 1–44 0	20 8
Hemoglobin (gm %)	5 0–13 0	8 4
MCHC (%)	22 4–30 0	27 0

Absorption of iron by subjects with excess iron stores (Figures 7 and 8)

Sixteen absorption studies have been performed on 9 patients with the excess iron stores of idiopathic hemochromatosis Seven of these patients were males and two[7] were females past the menopause The comparative absorption of ferrous chloride and Fe^{59} labeled eggs was evaluated in one patient with hemochromatosis The absorption of iron before and after an intensive venesection program was measured in one patient with

[7] We wish to thank Drs Alexander Marble and Frank Gardner, Boston, Massachusetts for the opportunity to study these patients

hemochromatosis and only after venesection in another

It is evident from Figure 7 that patients with well-established untreated idiopathic hemochromatosis of long duration with elevated serum iron levels and saturated iron stores did not absorb either ferrous chloride or food iron to any greater extent than did normal subjects studied under similar conditions. Patients with hemochromatosis absorbed and incorporated into hemoglobin 0.5 to 11.1 per cent of Fe^{59} administered as ferrous chloride. This represented the absorption of 0.1 to 3.3 mg of the 24 to 41 mg of carrier iron administered. Almost all of the remaining iron was recovered in the feces. Additional iron beyond that incorporated into hemoglobin may have been absorbed by the two female patients studied (Figure 7 Studies 7 and 8). The total quantity of iron possibly absorbed by these two female subjects was 28 and 29.7 per cent or approximately 11 mg of iron by each patient.

Absorption of food iron by untreated patients with hemochromatosis was evaluated in two patients who received Fe^{59} labeled beet greens containing 2.8 and 4.8 mg of iron (Figure 7). Only 1.0 and 1.1 per cent of iron was absorbed and incorporated into hemoglobin. This represents absorption of a quantity of food iron which is essentially the same as that obtained in normal subjects. If all the unrecovered iron was absorbed and deposited in tissue, the total absorption could be increased to 22 and 18.1 per cent, or 0.6 and 0.9 mg of iron.

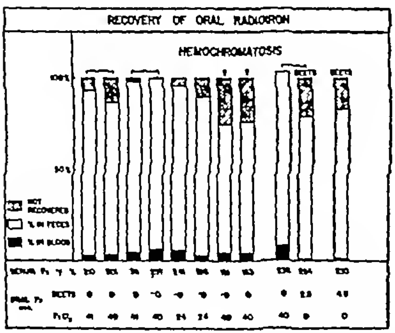

Fig. 7. Recovery of Radioiron in Patients with Hemochromatosis Following Administration of Ferrous Chloride or Labeled Beets

These patients had not been venesected. The symbol ♀ identifies a female patient.

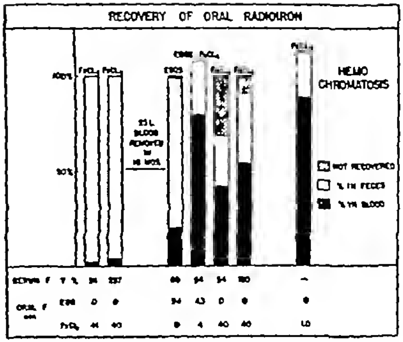

Fig. 8. Recovery of Radioiron Before and After Intensive Venesection Therapy of a Patient with Hemochromatosis (Studies 1 Through 6)

Recovery was measured in a second patient (study 7) only after multiple venesections.

Absorption of Fe^{59} labeled eggs was not evaluated in any untreated patient with hemochromatosis. However such a study was completed in one subject with hemochromatosis after the removal of 25 liters of blood over an 18-month period (Figure 8). Multiple iron absorption studies were completed before and after the venesection program. Initially the serum iron ranged between 235 and 311 micrograms per 100 ml. When studied at this stage he absorbed and incorporated into hemoglobin 4.7 to 6.2 per cent of a 40-mg dose of ferrous chloride. Ninety four per cent of the dose was recovered in stools. After extensive venesections during which period his hemoglobin remained at essentially normal levels and approximately 13 gm of iron were removed from his stores his serum iron was reduced to 60 micrograms per 100 ml. At this time he absorbed and used 20.2 per cent of 9.4 mg of food iron equivalent to the absorption of 1.9 mg of iron. This result approximates closely that obtained in subjects with iron deficiency who received labeled eggs. In contrast when Fe^{59} was administered as ferrous chloride along with nonradioactive eggs he absorbed and incorporated

initially [illegible] 8[illegible]4 per cent or 67 mg of an 80 mg dose of iron. [illegible] the serum iron [illegible] 51 micrograms per 100 ml, he absorbed and used [illegible]2 per cent or 15.2 mg of a 40 mg dose of ferrous chloride. The additional 20 per cent not recovered, if absorbed and added to tissue stores, would increase the total iron absorbed under these conditions to 2[illegible] mg. Without any additional venesections the serum iron gradually increased to 120 micrograms per 100 ml. At this time he absorbed and used 54.5 per cent, or 22 mg of a 40 mg dose. Most of the remaining iron was recovered in the feces except for 9 per cent, some of which may have been absorbed and deposited in tissue stores.

A second patient with hemochromatosis was studied only after extensive venesections.[4] He received Fe^{59} without added carrier iron, and absorbed and utilized 88.5 per cent of the administered iron for the formation of hemoglobin. The remainder was recovered in stools.

In summary, neither beef iron nor the iron salt, ferrous chloride, is absorbed to any demonstrably greater extent by patients with well-established untreated hemochromatosis than by normal subjects. An intensive venesection program will significantly lower the serum iron and remove iron from the body stores. Under these circumstances absorption of both ferrous chloride and egg iron is greatly increased, approximating that usually encountered in iron deficient subjects.

DISCUSSION

Our studies indicate that normal subjects, iron-deficient patients, and patients with idiopathic hemochromatosis absorb ferrous chloride more readily than food iron. In many of these studies a solution of ferrous chloride was administered to the subject in a quantity that considerably exceeded the amount of iron usually eaten by an individual in a single average meal. Chemical or physical substances that might decrease iron absorption were excluded since the patients were fasting. In most cases a supplementary reducing agent such as ascorbic acid was given with the ferrous chloride. There is evidence that the administration of ascorbic acid may increase the absorption of ferrous iron salts (19). However, in the present study there was no apparent difference between the absorption of iron by 7 subjects[5] who did not receive supplementary ascorbic acid and comparable subjects who received this reducing agent with ferrous chloride. No systematic comparison was made of the absorption of ferrous iron salts administered to the same patient with and without a reducing agent.

Iron in considerable excess of the daily loss of 1 mg may be absorbed when ferrous chloride is administered to normal subjects under the described experimental conditions. These results agree with those reported by Dubach, Callender, and Moore (27). Within the limitations imposed by inaccuracies in stool collection our data may be interpreted as lending support to their suggestion that normal subjects may absorb and deposit in tissue stores additional iron beyond that which is incorporated into hemoglobin.

Normal subjects absorbed much less iron after a single feeding of radioiron labeled eggs or vegetables. These observations were obtained under physiological conditions more closely approximating the ingestion of an average meal containing 5 mg of iron. Only 0.3 mg of iron at best, or approximately one-third of the daily iron requirement was absorbed and incorporated into hemoglobin. This observation agrees with previously reported studies (26). Although fecal recovery data suggest that additional iron in food may possibly be absorbed and deposited in body stores, the limited absorption of food iron observed supports the suggestion (26) that the daily adult requirement of 12 to 15 mg of dietary iron recommended by the National Research Council (32) may be barely sufficient to maintain the body iron stores.

The present studies indicate that patients with iron deficiency usually absorb significantly more food iron than normal subjects. However, the amount of iron absorbed from food was not much greater than the quantity lost daily from the body in the absence of bleeding or pregnancy. Moore and Dubach, on the other hand, found only a few iron-deficient subjects who absorbed more food

[4] This patient was studied through the cooperation of Dr. Wayne Rundles, Duke University School of Medicine.

[5] These 7 subjects include 5 normal subjects, 1 iron deficient patient, and 1 patient with idiopathic hemochromatosis.

iron than did normal individuals although the co-administration of a reducing substance such as ascorbic acid significantly increased the quantity of iron absorbed (26) In our studies markedly limited absorption of food iron was observed in an iron-deficient patient with a subtotal gastrectomy Iron as ferrous chloride was as readily absorbed by this patient as by the other iron-deficient patients Partial gastrectomy may have influenced the absorption of food iron since there is some evidence that dietary iron is ionized, reduced to the ferrous form and better absorbed at an acid pH (33–37)

The limited absorption of food iron in these studies suggests that it would be difficult for an iron-deficient patient to replenish his stores by diet alone Supplementary medicinal iron seems indicated for individuals who undergo chronic blood loss, have increased physiological requirements or have an absorptive defect due to an operative procedure *e g* subtotal gastrectomy, or steatorrhea (7 26 38 39) Therapy should be continued after the hemoglobin level has been restored to normal since it has been shown that depleted iron stores are not easily reconstituted (40)

A different situation is observed in hemochromatosis Although our studies indicate that neither ferrous chloride nor food iron is absorbed to any greater extent in well-established hemochromatosis than in normal subjects it appears necessary to assume that excess iron must be absorbed during the developmental phase of this disease By no other means can one satisfactorily explain the huge iron stores found in a patient with idiopathic hemochromatosis This assumption is perhaps supported by our observation that absorption of both ferrous chloride and food iron was markedly increased in patients with hemochromatosis after they had undergone an intensive venesection program After 12 to 15 grams of iron had been removed by weekly or biweekly 500-ml venesections the absorption of iron was approximately that seen in iron deficiency It is significant that this increased absorption was observed even after the serum iron concentration had returned to normal (120 micrograms per 100 ml)

Studies showing increased radioiron absorption in younger patients with hemochromatosis have been reported recently which lend support to the concept that iron absorption must be increased during the developmental stage of idiopathic hemochromatosis (41–43) External measurements indicated most of the absorbed iron was stored in the liver (43 44) Increased absorption of a lesser degree has also been observed in three older female patients (43) Of 9 patients with hemochromatosis in our studies the two women were the only patients who appeared to absorb an increased quantity of iron. Menstrual loss of blood may have had an effect similar to that of repeated, small venesections so that full development of the disorder was delayed Our studies after venesection as well as those of Peterson and Ettinger (41) do show that extensive blood loss will modify the absorption of iron even after hemochromatosis has fully developed Increased absorption may then occur even with a normal serum iron concentration These results suggest iron stores may reaccumulate in idiopathic hemochromatosis unless vensections are continued at intervals as recommended by Finch and Finch (45)

Although the present study has demonstrated greater absorption of iron salts than of food iron it does not provide a clear explanation why this occurs Multiple studies in normal subjects evaluating the comparative absorption of labeled egg iron and of ferrous chloride alone and with non labeled eggs suggest that the presence of egg will decrease the absorption of the iron salt. This decreased absorption may be related to the solid content of the test meal (46) or formation of an insoluble compound of iron with a chemical constituent of egg or bread Hegsted, Finch and Kinney (47) have shown that a high concentration of added phosphate will decrease absorption of iron by rats on a corn grit diet, presumably by formation of an insoluble iron phosphate. Similarly, soluble phytates may also interfere with iron absorption (46 48) Conversely rats on a corn grit diet with added iron will absorb large amounts of iron and produce progressive hemosiderosis of the tissues (49) Studies by Hegsted Finch, and Kinney indicate that the low level of dietary phosphate attained with a corn grit diet was primarily responsible for increased absorption of iron (47) Such increased absorption of iron observed on a phosphate-deficient diet with excess iron might account for development of dietary hemosiderosis

observed in malnourished pellagrins in South Africa (50, 51)

The influence of phosphates on iron absorption may explain the present observations that egg iron was poorly absorbed and that addition of egg decreased absorption of ferrous chloride Halkett, Peters, and Ross found that egg yolk iron is in the ferric state and is strongly complexed to the phosphate of yolk phosphoproteins (52) Formation of such an iron phosphate complex occurs both in the biological production of eggs and when iron is added to eggs *in vitro* They further observed that egg yolk iron is not removed by peptic digestion and acidity unless a reducing agent is present It appears egg iron is not readily available for absorption and it is not surprising that so little was absorbed in the present study as well as in previous animal studies (53) In contrast it has been shown that a 10 to 20 fold increase in the absorption of food (egg) iron generally occurs in iron-deficient subjects when large amounts of ascorbic acid are administered with the iron (7, 26) This effect is presumably dependent upon the reduction of iron to the ferrous form and may be accomplished by other reducing substances in food

It is of interest to consider how results of the present studies may relate to the theory that the intestinal mucosa is an important regulator of iron absorption (24, 54–57) According to Granick (56), iron is transferred from the intestinal lumen to blood by a protein, apoferritin, present in cells of the intestinal mucosa. Iron is taken up by the mucosal cells until all apoferritin is converted to ferritin No more iron may then be absorbed until ferritin has given up iron to plasma This is the concept of the "mucosal block" originally suggested by Hahn, Bale, Ross, Balfour, and Whipple (24)

Dubach, Callender, and Moore (27) have presented evidence that this block is at best a partial one and that in certain conditions, such as refractory anemia, pernicious anemia in relapse, or hemolytic anemia, the mucosal block does not prevent iron from being absorbed in spite of adequate body iron stores We have observed a similar increase in iron absorption in thalassemia minor and in renal anemia (44) Moreover, it would appear that the block must fail significantly during the developmental stage of idiopathic hemochromatosis and again in this disease after an extensive course of phlebotomies Other examples of the alteration of the mucosal block in animals and men have been discussed previously in relation to factors that influence absorption of iron

Finally, our data in normal subjects, as well as those of others (27) suggest that the "mucosal block" does not prevent absorption of an increased quantity of iron salt when it is administered in a single feeding to a fasting subject under optimal conditions, *i e*, as a solution of ferrous chloride with ascorbic acid, or when given in large quantities (4) Such excess iron absorption has also been observed after average doses of iron salts have been orally administered for a period of years (58, 59)

SUMMARY AND CONCLUSIONS

1 The iron salt, ferrous chloride, is absorbed far more readily and in greater quantity by normal subjects and by patients with deficient and excess iron stores than is iron present in certain foods (eggs, vegetables)

2 Egg and vegetable iron are not absorbed sufficiently to supply iron in the face of increased loss or increased physiological requirements

3 Absorption of certain food iron and ferrous chloride in patients with well-established hemochromatosis is approximately equal to that observed in normal subjects However, it can be assumed that excess quantities of iron must be absorbed during the developmental phase of this disease

4 After removal of blood by multiple venesections absorption of ferrous chloride and egg iron by patients with idiopathic hemochromatosis is markedly increased

5 Absorption of iron salts is significantly influenced by dietary factors which may modify the form and solubility of iron in the lumen of the gastrointestinal tract

6 Further evidence has been presented to support previous data in the literature which indicated that the "mucosal block" to iron absorption is only relatively complete and may not uniformly prevent the excess accumulation of iron in the body

ACKNOWLEDGMENTS

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AN EVALUATION OF THE SINGLE INJECTION THIOSULFATE METHOD FOR THE MEASUREMENT OF EXTRACELLULAR WATER[1]

By LEON C. CHESLEY and ABRAHAM LENOBEL

(From the Department of Obstetrics and Gynecology State University of New York College of Medicine at New York City and the Kings County Hospital Brooklyn N Y)

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The purpose of this paper is to examine the validity of the single injection thiosulfate method, proposed by Cardozo and Edelman (1) for the measurement of the extracellular fluid volume. The conclusion is that the method does not give a valid estimate of the extracellular fluid volume and probably does not even measure the volume of distribution potentially available to thiosulfate.

Cardozo and Edelman gave an accurately known amount of sodium thiosulfate (about 10 gm. of $Na_2S_2O_3$ 5 H_2O in 10 per cent solution) by intravenous injection over a period that varied from 7 to 16 minutes They then collected venous blood samples at intervals and plotted the logarithms of the serum thiosulfate concentrations against time. The points usually fell on a straight line, this was extrapolated back to zero time, which arbitrarily was set as the moment the infusion had been started If the points did not fall on a straight line, the test was discarded because of the variable clearance rate of the thiosulfate The extrapolated value for serum concentration of thiosulfate (P_0) is presumed to be what the concentration would be if the thiosulfate were instantaneously injected and evenly distributed in its final volume of dilution This extrapolated P_0 is then divided into the total amount of thiosulfate injected to calculate the volume of distribution

Aside from the fact that virtually none of the thiosulfate has been injected at this zero time, the method is based upon rather questionable assumptions which will be discussed later

In some of the evaluations of the single injection method that follow comparisons were made between the volumes of distribution of thiosulfate and sucrose as calculated by different methods. The other procedures used were the infusion/slope method devised by Schwartz (2) and the calibrated infusion (IV minus UV) method of Deane, Schreiner, and Robertson (3)

MATERIAL AND METHODS

All experiments were done with hospitalized women. While they were not "normal" they were selected. Patients with any discernible cause for disturbance in hydration, such as fever renal disease or heart disease, were excluded. All patients were less than age 50 years and most were in the third and fourth decades of life. Nearly all patients were fasting the day of the test. The few exceptions are those patients in whom simultaneous measurements were made of the volumes of distribution of sucrose and thiosulfate.

Reagent grade sodium thiosulfate ($Na_2S_2O_3$) was made up as a 6.7 per cent solution in either isotonic saline or 5 per cent dextrose. This gives the same thiosulfate concentration as Cardozo and Edelman used in their 10 per cent solutions of the hydrated salt. The solution was sterilized by Seitz filtration and given intravenously from a calibrated buret. The dose was roughly 0.5 ml. per pound of body weight and was given over a period of from 8 to 14 minutes. Collections of venous blood were begun 25 minutes after the end of the infusion and five samples were taken over the next 75 minutes. When the volume of distribution was measured by the infusion/slope method, the priming dose was followed by constant infusion of more of the same solution at the rate of 1.05 to 1 10 ml. per minute for periods of 2 to 3 hours. The constant infusion was given by a Bowman pump which was recalibrated after each use.

The sucrose solutions used in measuring the volume of sucrose distribution were made up in 8 per cent concentration in either isotonic saline or 5 per cent dextrose. (The latter gives an appreciable blank in the chemical method for measuring sucrose)

In the calibrated infusion method this solution was given intravenously at the rate of 1 05 to 1 10 ml. per minute. Half hourly collections of venous blood and of urine were begun 2 hours after the start of the infusion. After the collection of 3 or 4 blood and urine samples the thiosulfate was given for the single injection procedure.

In measuring the volume of sucrose distribution by the

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infusion/slope method, a priming dose of 8 per cent sucrose was followed by more of the same solution, as described for the administration of thiosulfate. Usually, simultaneous measurements were made of the volumes of sucrose and thiosulfate distribution, with sucrose and thiosulfate combined in the same solution In the infusion/slope procedure, a minimum of three venous blood samples were taken during the constant infusion, the first being obtained about 90 minutes after the beginning of the infusion. Five more venous blood samples were collected over the 100 minute period following the end of the infusion. In both the single injection method and the infusion/slope method the test was discarded in any case where the logarithms of the serum thiosulfate or sucrose concentrations, plotted against time, were not closely fitted by a straight line. Also no calculations were made from the data obtained by the infusion/slope method unless the serum concentrations of thiosulfate and sucrose had attained constancy

The solutions injected were hypertonic, but the volumes were small in comparison to the volumes of distribution, being about 1 per cent in the single injection procedure. In the constant infusion methods the rate of administration was 1 07 ml per minute, so that in 3 hours the total volume given was of the order of 2 to 3 per cent of the volume of distribution Meanwhile renal excretion and metabolism of the solutes (except for so-ium chloride) kept pace with the infusion rate in the ises studied by the infusion/slope method. Further-ore, the procedure was the same in all experiments ex-:pt that solutions were sometimes given in 0 9 per cent iline and sometimes in 5 per cent dextrose.

For chemical analyses, two samples of the infusion olution were diluted (1 1,000 for thiosulfate and 2 000 for sucrose), urine samples were suitably di-ited (usually 1 500 or 1 1,000) and serum samples ere prepared by precipitating the proteins For thio ilfate the serum was diluted 5 times in the protein pre-pitation by tungstic acid and for sucrose it was di-ited 10 or 20 times in precipitating the proteins by zinc ydroxide. All samples were analyzed in duplicate or uadruplicate. Thiosulfate was measured by the iodine tration method of Brun (4) and sucrose by the resorci-ol method of Roe, Epstein, and Goldstein (5), using a eckman DU spectrophotometer Blood, urine, water nd reagent blanks were analyzed with all measurements When sucrose infusions were given in 5 per cent dextrose, an aliquot of the original dextrose solution was diluted 1 2,000, as was the infusion solution. These dextrose blanks were carried through the sucrose analyses and their optical densities were substracted from the optical densities of the infusion samples This correction factor averaged about 4 per cent of the readings of the infusion samples

RESULTS

Experimental alteration of the renal clearance rate

The first experiments with the single injection method were designed to find what effect changing the slope of the exponential disappearance rate of serum thiosulfate would have upon P_0 and, therefore, upon the calculated volume of thiosulfate distribution The slope of the exponential curve was changed in two ways (*a*) by pre-medication with carinamide, which partially blocks the renal tubular excretion of thiosulfate (6), and (*b*) by intravenous theophylline ethylenediamine, which increases the glomerular filtration rate (7) and therefore the renal excretion rate of thiosulfate More than half of the experiments could not be used because of the irregular effect of the drugs over the 100-minute period following the infusion of the thiosulfate (This irregularity is reflected in a non-linear plot of the logarithms of the decreasing serum thiosulfate concentrations against time Cardozo and Edelman wrote that such cases can not be used, for obvious reasons) By trial and error the best dose of carinamide was found to be 4 gm given at 6 00 A M and 2 gm given at 9 00 A M, with the thiosulfate infusion given at 10 00 A M In the theophylline ethylenediamine experiments, 0.25 gm of the drug was given intravenously about 10 minutes before the thiosulfate infusion and this was followed with a continuous infusion of another 0 25 gm in 250

TABLE I

Experimental alteration of the clearance rate of serum thiosulfate by carinamide or theophylline, showing the times at which the experimental curves intersect with the curves found without medication and the percentage difference in calculated thiosulfate space resulting from the change in extrapolated P

Experiment	1a	1b	2	3	4	5	6	7	8	9
Minutes after zero time at which curves cross	29	30	2	No	18	21	27	29	16	36
Percentage difference in thiosulfate space resulting from changed excretion rate	10	24	7	7	19	18	11	13	17	30

ml of 5 per cent dextrose over the 100-minute period of the test.

Nine successful experiments were done in fasting women selected with special care as to "normality" In all cases the thiosulfate was given in 5 per cent dextrose. The order in which the experiments were done was varied from case to case and the tests were done on successive days in any one patient. All exponential disappearance curves for serum thiosulfate were fitted by the method of least squares to ensure objectivity If the single injection method is valid the disappearance curves should intersect at zero time, that is P_0 should be constant because the amount of thiosulfate given was constant and the potential volume of distribution should not vary much from test to test (see Discussion)

Table I shows that the disappearance curves did not intersect at zero time in one case the lines did not cross at all and in the majority of cases the intersections occurred at 20 to 30 minutes after zero time. The experimental alteration of the renal clearance rate of thiosulfate changed the calculated volume of distribution in every case the change being from 7 to 30 per cent One of the experiments is shown in Figure 1, where the lines cross at 29 and 30 minutes In this particular case, the thiosulfate space calculated from P_0 with carinamide is 138 per cent of that calculated from P_0 with theophylline ethylenediamine, the calculated volumes were 11.7 and 8.5 liters, respectively This is the only experiment, in more than 20, in which three good disappearance curves were obtained. In the other eight cases reported there were two good curves but the third with either carinamide or theophylline ethylenediamine departed from linearity on the semilog plot. Of the eight, five compare curves with no medication to curves with carinamide and three compare curves with no medication to those with theophylline ethylenediamine.

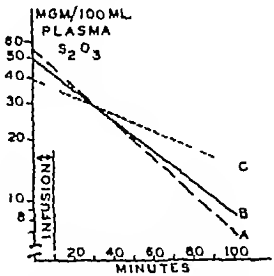

FIG. 1 THE EFFECT OF ALTERING THE RENAL EXCRETION OF THIOSULFATE UPON THE VALUE OF EXTRAPOLATED P

A = pre-medication with theophylline ethylenediamine B = basal measurement with no medication, C = premedication with carinamide. Note that P is a function of the clearance rate of serum thiosulfate.

It would appear that the value for P_0 is a function of the clearance rate of thiosulfate Experimental alteration of the renal clearance rate changes P_0 and results in changes in a predictable direction of the calculated volume of thiosulfate distribution Decreasing the slope of the curve increases the calculated volume of distribution while increasing the slope of the curve decreases the calculated volume.

Comparison of thiosulfate spaces measured by the infusion/slope method to those calculated from the single injection method

The single injection method was compared with the infusion/slope method in six fasting patients with alternation of the method first applied In any one patient the tests were done on successive days All solutions were given in 0.9 per cent saline.

The pairs of measurements did not check. In five of the six cases the volume of distribution for thiosulfate was considerably greater as measured by the infusion/slope method than as estimated from the single injection method The results in Table II show that the average discrepancy was 30 per cent and ranged from 0 to 53 per cent.

Comparison of the thiosulfate space as estimated by the single injection method to the volume of sucrose distribution

In 18 patients the sucrose space was measured by the calibrated infusion method and immediately thereafter a single injection of sodium thiosulfate was given for the estimation of the volume of thiosulfate distribution. No experiment is included unless equilibrium of sucrose was established (that is essentially constant volumes of

TABLE II

Comparison of measurements of the thiosulfate space by the methods of Cardozo and Edelman and of Schwartz and of the slopes of the serum thiosulfate disappearance curves in the two methods

Case	1	2	3	4	5	6	Mean
Body weight, *Kg*	55 0	65 0	69 1	63 7	56 8	50 9	
Thiosulfate space							
Cardozo and Edelman	9 8	7 8	7 9	8 2	8 8	10 6	8 85
Schwartz	12 0	11 9	11 5	11 5	11 6	10 6	11 50
Ratio, Schwartz to Cardozo and Edelman methods	1 22	1 53	1 46	1 40	1 32	1 00	1 30
Ratio, disappearance curve slopes, Cardozo and Edelman to Schwartz methods	1 60	1 96	2 14	1 86	1 67	1 87	1 85

distribution in successive periods) In all but two cases the solutions were given in 5 per cent dextrose The calculations from the data for the single injection method gave apparent volumes of distribution for thiosulfate that averaged about 35 per cent less than the sucrose space, as also was found by Ikkos (8) The apparent volume of thiosulfate distribution, as calculated by the single injection method, bore no constant relationship to the volume of distribution of sucrose, as the scattering of points in Figure 2 shows Of course, sucrose may not measure the extracellular fluid volume but under the conditions of the experiments a steady state had been attained and a reproducible space had been measured (This often was not true in Ikkos' experiments, for in about 80 per cent of his cases the apparent volume of sucrose distribution increased steadily with time Arbitrarily, he took the apparent volume of distribution at 150 minutes for comparison with apparent volumes of thiosulfate distribution)

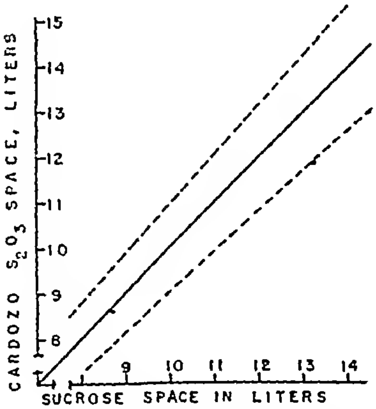

FIG 2. COMPARISON OF THE THIOSULFATE SPACE, AS CALCULATED BY THE CARDOZO AND EDELMAN SINGLE INJECTION METHOD, TO THE SUCROSE SPACE MEASURED ALMOST SIMULTANEOUSLY

The solid line is the line of identity and the broken lines represent plus and minus 10 per cent.

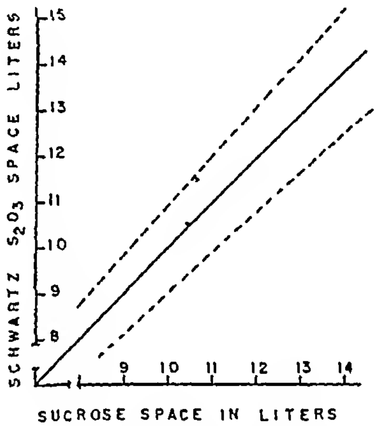

FIG 3 COMPARISON OF THE THIOSULFATE SPACE, AS MEASURED BY THE SCHWARTZ INFUSION/SLOPE METHOD, TO THE SUCROSE SPACE MEASURED SIMULTANEOUSLY

The solid line is the line of identity and the broken lines represent plus and minus 10 per cent.

Comparison of the thiosulfate space measured by the infusion/slope method with the volume of distribution of sucrose

Measurements of the volume of distribution of thiosulfate, made with the infusion/slope method, were compared with simultaneous measurements of the volume of sucrose distribution in 14 patients In nine of these the volume of sucrose distribution

TABLE III

Comparison of thiosulfate and sucrose spaces measured simultaneously by the infusion/slope method of Schwartz

Case:	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sucrose space, *l*	10 4	13 7	9.0	10 9	10 6	12.5	10.2	10.5	13 8	12.5	10 9	9.2	12.4	9 8
Thiosulfate space, *l*	10 5	14.3	9 4	10.2	11 5	11 1	11 6	8 9	11.5	15 1	8 6	11.3	9 4	15 1
Percentage deviation of thiosulfate space from sucrose space	1	4	5	6	8	11	14	15	16	21	21	23	24	54

was measured both by the infusion/slope method and by the calibrated infusion method, in the other five one or the other of the methods was used

Figure 3 shows that there was no consistent relationship between the thiosulfate and sucrose volumes of distribution although the average difference was very small Relative to the volume of sucrose distribution the thiosulfate space varied from plus 54 to minus 24 per cent, as shown in Table III While the average ratio is close to 1 0 the wide scatter of the points in Figure 3 indicates that this may be fortuitous

Comparison of volumes of sucrose distribution measured simultaneously by the infusion/slope and calibrated infusion methods

Inasmuch as the calculations of the volume of thiosulfate distribution did not agree when the

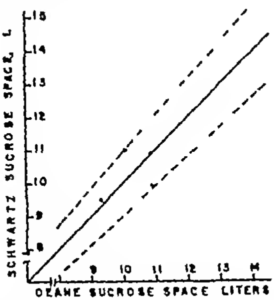

FIG. 4 COMPARISON OF SIMULTANEOUS MEASUREMENTS OF THE SUCROSE SPACE MADE BY THE SCHWARTZ INFUSION/SLOPE METHOD AND BY THE DEANE, SCHREINER, AND ROBERTSON CALIBRATED INFUSION (IV MINUS UV) METHOD

The solid line represents identity and the broken lines plus and minus 10 per cent.

single injection and the infusion/slope methods were applied successively in individual patients it is of interest to compare the infusion/slope method to some other procedure Therefore simultaneous measurements of the volume of sucrose distribution were made by the infusion/slope and the calibrated infusion methods Here the sucrose was given in 0.9 per cent saline and the priming dose of sucrose was added in the IV' factor of the IV minus UV formula of the calibrated infusion method.

As Figure 4 shows these methods, quite different in principle, gave very similar volumes for the sucrose distribution All but 1 of the 10 pairs of measurements checked within 10 per cent The points shown in the graph are symmetrically distributed about the line of identity and the average difference between the simultaneous measurements is zero Here the average ratio of 1 0 may not be fortuitous for in contrast to the comparison of volumes of thiosulfate and sucrose distribution the pairs of measurements check closely

DISCUSSION

The apparent advantage of the single injection method lies in its circumvention of the constant infusion and urine collections However its theoretic bases do not seem sound Thiosulfate is known to enter the red blood cells (1) and if it enters these cells it may enter others Gilman Philips and Koelle (9) found that in dogs about one-fourth of the injected thiosulfate cannot be recovered and they concluded on good evidence, that most of the irretrievable fraction disappeared during and just after the infusion The incomplete recovery was confirmed for man, by Cardozo and Edelman This together with the odor of the urine after thiosulfate injection suggest that

thiosulfate is metabolized in the body Inasmuch as it is relatively stable in blood, *in vitro*, such metabolism probably occurs in cells

The extrapolation of the decremental curve back to zero time implies questionable assumptions One assumption is that thiosulfate disappears at the same exponential rate from the beginning of the infusion to the end of the blood collections (100 minutes) The assumed starting point for this exponential decrement in plasma concentration is based not upon the amount already injected at zero time, but upon the total amount to be injected over the next 10 or more minutes This does not seem reasonable Also, even when such injections are given within seconds, the early portion of the decremental curve is not adequately described by the course of the exponential limb, as shown by Sheppard, Overman, Wilde, and Sangren (10) and by Sapirstein, Buckley, and Ogden (11) Another assumption is shown graphically in the first figure in the paper by Cardozo and Edelman The early portion of the decremental curve lies above the extrapolated exponential limb (as in all such cases), Cardozo and Edelman factor this early portion of the curve into two straight (exponential) lines by the standard procedure of subtracting the extrapolated limb from the observed points From this they draw the conclusion that two processes are going on in the early minutes (*a*) equilibration of the thiosulfate throughout plasma and interstitial fluid, and (*b*) clearance of thiosulfate at a uniform exponential rate, by renal excretion and metabolism However, the rapid component of the decremental curve, in man, may represent the immediate loss of thiosulfate, which Gilman, Philips, and Koelle found in dogs If the steep curve represents equilibration of thiosulfate throughout all of extracellular water, as Cardozo and Edelman suggest, the distribution must occur with almost incredible rapidity—within 10 minutes, as they say In view of the time (30 to 120 minutes) required for sucrose to attain its maximum volume of distribution in normal subjects, it does not seem likely that thiosulfate would become evenly distributed in so short a time The diffusion coefficients can not be specified exactly because of concentration effects Cardozo and Edelman cited 0 68 cm^2 per day for thiosulfate and Raisz, Young, and Stinson (12) cited 0 55 cm^2 per day for sucrose, the source in both cases is the International Critical Tables

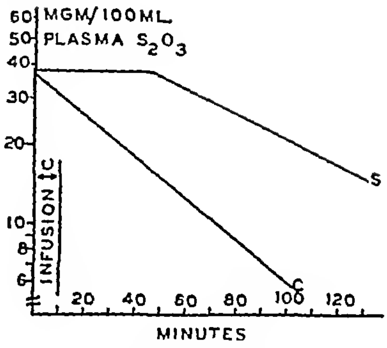

FIG 5 COMPARATIVE RATES OF CLEARANCE OF SERUM THIOSULFATE IN THE SCHWARTZ INFUSION/SLOPE METHOD (S) AND CARDOZO AND EDELMAN SINGLE INJECTION METHOD (C)

The constant infusion of the Schwartz method had gone on for 2 hours before the beginning of the time shown in the graph, allowing time for the establishment of a steady state. "Infusion C" refers to the period of infusion in the Cardozo and Edelman method.

One observation is consistent with the interpretation that the steep component may represent immediate loss of thiosulfate Figure 5 and the last line in Table II show that the slope of the serum thiosulfate disappearance curve was nearly twice as great in the single injection procedure as in the infusion/slope method This suggests that the prolonged infusion of the latter method may have "saturated" some space not so "saturated" in the much shorter period of the single injection procedure, where thiosulfate may still be leaving the blood stream by diffusion into extravascular fluid throughout the whole course of observation Possibly this "saturation" occurring in the prolonged period of constant infusion represents the attainment of concentration equilibrium between plasma and extravascular fluid available to thiosulfate There are other possible factors in the slower rate of clearance from the plasma, after prolonged infusion One might be increased feedback from extravascular sources, this, again, would suggest that in the single injection method the extravascular sources had not been brought into equilibrium with plasma water Another possibility is that the rate of metabolism of thiosulfate slows down after prolonged infusion

If a large proportion of the injected thiosulfate does disappear quickly, in man, then obviously the

dividend in the equation for the single injection method is too great and of itself should give calculated volumes of distribution that are too large However the volume calculated from this equation averages 30 per cent less than the measurements of the thiosulfate space by the infusion/slope method This suggests that the divisor (P_0) in the equation for the single injection method is too large and in even greater error than is the dividend This could result from the failure of thiosulfate to diffuse into and attain equilibrium in its potential total volume of distribution within the short period of time encompassed in the test Thus at least two variable and partially compensatory errors fortuitously result in calculations of an apparent thiosulfate space that often falls within the range described as normal for the volume of distribution of inulin

The experimental alteration of the renal clearance of thiosulfate changes P_0 and therefore the apparent volume of thiosulfate distribution as calculated in the single injection method Since this work was completed Peterson O'Toole and Kirkendall (13) have reported marked variations in the sucrose space of man during a 24-hour period. Inasmuch as the present experiments in which the renal clearance rate of thiosulfate was varied, were done on successive days the assumption that the space should be nearly constant from day to day may be in error In the three patients studied in regard to such reproducibility there was less than 5 per cent variation in the calculated volume of the thiosulfate distribution from day to day No more cases were studied because of the relative constancy reported for repeated estimations by Cardozo and Edelman (1), Ikkos (8), Raisz Young and Stinson (12) Becker and Joseph (14) and others The reproducibility seems to be within the limits of analytic error Even if it were not changing the renal clearance rate of thiosulfate has invariably changed the calculated volume of distribution in a predictable direction

SUMMARY

Experimental alteration of the renal excretion rate of thiosulfate, by pre medication with carinamide or theophylline ethylenediamine, changed significantly the volume of thiosulfate distribution as calculated by the single injection method.

The volume of thiosulfate distribution as measured by the infusion/slope method averaged 30 per cent greater than the space calculated by the single injection method.

The thiosulfate space measured by the infusion/slope method or calculated by the single injection method, bore no constant relation to the volume of sucrose distribution

Simultaneous measurements of the sucrose space by the infusion/slope and by the calibrated infusion methods gave consistent results

The failure of the single injection method to give thiosulfate spaces even approximating those measured by the infusion/slope method strongly suggests that the single injection method does not measure the volume of thiosulfate distribution

It is concluded that the single injection method is not sound theoretically and that apparent volumes of distribution calculated from P_0 have no significance as spaces They only fortuitously come close to volumes measured by sucrose or inulin because of multiple variable and partially compensatory errors

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TABLE I

Physical status of dogs with chronic aortic-caval fistulas

Dog No.	Weight (Kg) Before	Weight (Kg) After	Fistula duration (days)	Remarks
1	13.1	12.6	37	No edema, ascites or evidence of circulatory stress
2	12.6	13.0	30	Hind leg edema No ascites or evidence of congestive heart failure
3	15.5	21.4	55	Much edema of all extremities and ascites, tissue loss
4	11.8	11.2	49	Slight ascites and leg swelling
5	12.3	11.1	23	No ascites, edema or evidence of circulatory stress
6	10.0	13.8	40	Stormy history with marked ascites, edema of all extremities
7	17.3	15.4	385	Mild ascites and edema of front and hind extremities
		17.8	425	Marked ascites and edema of all legs with considerable recent weight gain
8	17.3	16.8	69	No ascites, slight edema
9	13.6	21.8	69	Considerable ascites and edema of front and hind legs Pulmonary edema at death on 153rd day
10	14.6	14.1	70	Considerable edema of all legs
11	18.2	17.2	70	Pulmonary edema
12	17.3	20.9	150	Marked ascites, peripheral edema

was accomplished by premedication with 3 mgm per Kg of morphine sulfate, followed by 0.25 cc. per Kg of a 1 1 mixture of Dial urethane and pentobarbital. Arterial oxygen saturation was maintained by a demand valve apparatus connected to an oxygen tank, phasic intrapleural pressure was recorded with an optical segment capsule connected to a trocar in the left intrapleural space near the heart, and these pressures were used to correct the simultaneously recorded left ventricular end-diastolic pressure. Procaine amide (200 mgm. in 50 cc. saline) was given to abolish sinus arrhythmia in some of the animals (noted in Table II) The methods used to analyze the data were as follows 1) Stroke volume index in cc. per M^2 (stroke volume/surface area M^2), 2) stroke work index in gm M per M^2 (stroke volume index $\times$ (mean arterial pressure $-$ left ventricular end-diastolic pressure) $\times$ 13.6), 3) cardiac work index in gm M per M^2 per min. (cardiac index $\times$ (mean arterial pressure $-$ left ventricular end diastolic pressure) $\times$ 13.6), and 4) total peripheral resistance (mean arterial pressure $\times$ 100/cardiac output in cc.)

After control observations of cardiac output and pressures had been completed, either 0.045 or 0.025 mgm. per Kg of Lanatoside C was given intravenously as a single dose and at the end of 30 to 60 minutes the observations were repeated. The larger amount of the drug is at

TABLE II

Effect of Lanatoside C in dogs with aortic-caval fistulas †

Dog No	L.V.E.D.P. B	L.V.E.D.P. A	C.I. B	C.I. A	C.W.I. B	C.W.I. A	S.V.I. B	S.V.I. A	S.W.I. B	S.W.I. A	M.A.P. B	M.A.P. A	T.P.R. B	T.P.R. A	H.R. B	H.R. A
1	32	35	6.5	6.8	4,150	6,080	62	60	40	54	79	101	2.1	2.5	104	113
2	15	12	4.3	4.7	3,390	5,000	59	55	45	59	71	90	2.8	3.3	74	85
3	19	17	4.3	5.1	4,250	5,960	34	39	33	45	91	103	2.7	2.6	129	132
4	22	26	8.1	7.2	6,640	6,250	66	68	54	59	82	90	1.8	2.2	122	106
5	19	13	10.5	7.9	8,430	7,070	69	53	55	47	78	79	1.4	1.9	152	150
6	37	37	9.6	8.8	2,814	3,638	58	56	17	24	58	67	1.0	1.3	167	157
7	26	22	11.0	7.6	10,580	9,040	76	77	73	91	97	109	1.4	2.2	145	99
7′	29	30	6.7	6.1	6,710	7,503	69	63	69	77	103	120	2.2	2.7	97	97
8	13	15	10.1	9.6	15,444	13,474	94	90	143	124	125	117	1.8	1.8	108	108
9*	28	26	7.6	7.7	5,374	6,180	55	59	40	48	80	85	1.3	0.9	138	130
10*	14	14	6.9	5.8	6,832	4,318	86	71	86	54	87	70	2.0	3.1	80	80
11*	28	29	6.7	6.5	6,370	6,365	39	44	38	42	98	102	1.7	2.2	170	150
12	26	17	6.9	8.3	5,161	5,983	50	69	37	49	81	70	1.4	1.0	138	120
Aver	24	23	7.6	7.1	6,620	6,682	63	62	56	60	87	93	1.8	2.1	125	117

† Measured and calculated parameters *before* (B) and 30 to 60 minutes *after* (A) the injection of Lanatoside C Left ventricular end-diastolic pressure mm Hg (L.V.E.D.P.) cardiac index (C.I.), cardiac work index (C.W.I.), stroke volume index (S.V.I.) stroke work index (S.W.I.) mean arterial pressure, mm Hg (M.A.P.), total peripheral resistance (T.P.R.), and heart rate (H.R.) The data under 7 and 7′ were obtained from the same animal on the 385th and 425th post-operative day The asterisk indicates those dogs receiving 0.025 mgm per Kg of Lanatoside C, all others received 0.045 mgm. per Kg Animals 1 to 7 were studied under Dial-urethane pentobarbital anesthesia and received procaine-amide to abolish sinus arrhythmia Animals 8 to 12 were trained and determinations were made using local procaine (1 per cent) subcutaneously at the site of needle puncture.

TABLE III

Effect of maintenance digitalization in dogs with aortic-caval fistulas *

Dog No.	Weight (Kg)		L.V E.D P		C.I.		C.W.I		S.V.I		S.W I.		M.A.P		T.P.R.		H.R.	
	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
8	16.8	16.8	13	4	10.1	6.7	15 444	7 470	94	61	143	68	125	86	1.8	1.8	108	110
9	21.8	19.5	29	21	7.6	7.1	5 374	8 015	55	44	40	50	80	104	1.3	1.8	138	160
10	14.1	13.6	14	9	6.9	4.5	6 832	4 957	86	45	86	49	87	89	2.0	3.5	80	100
11	17.2	17.2	28	32	6.7	6.3	6,370	4 660	39	45	38	34	98	86	1.7	1.9	170	140
12	20.9	20.9	26	33	6.9	7.9	5 161	3 700	50	66	37	31	80	68	1.4	0.9	138	120
Aver	18.2	17.6	22	20	7.6	6.5	7 836	5 760	65	52	69	46	94	87	1.6	2.0	127	126

* Animals 8, 9 10 and 11 maintained on Digitoxin for four days after initial digitalization with Lanatoside C studies made before and on fourth day after digitalization. Animal 12 studied before and one day after digitalization Abbreviations as in Table II

least 30 per cent of the lethal dose reported for some other cardiac glycosides (4) and is about twice the dose used per kilogram of body weight in the studies by Stead, Warren, and Brannon (5) on human subjects in congestive heart failure. This amount of the drug is an effective dose, as indicated by the work of Li and Van Dyke (6) In one unanesthetized animal the observations were repeated the next day and in four others, they were repeated after four days of digitalization. The four animals received daily maintenance doses of digitoxin, administered intramuscularly in amounts equivalent to one tenth of the original digitalizing dose of Lanatoside C (Table III)

RESULTS

In the group of dogs with fistulas hemodynamic studies were made 23 to 425 days after creation of the aortic-caval fistula The condition of the dogs and their hemodynamic status varied considerably (Table I) Some gained a considerable amount of weight with ascites and edema of all extremities while others gave no external evidence of circulatory stress or insufficiency Table II presents the data obtained in the group with fistulas both anesthetized (1–7) and unanesthetized (8–12) before and 30 to 60 minutes after the intravenous administration of Lanatoside C. It can be seen that before digitalization the average work load of these hearts was quite high The left ventricle maintained an average cardiac index of 7.6 liters per minute, a cardiac work index of 6,620 gm meters and a stroke volume index of 63 cc. The carotid pulse pressure averaged 60 mm Hg (data not shown) despite a heart rate of 125 per minute and a high left ventricular end diastolic pressure of 24 mm. Hg In some dogs previous measurements indicated that the extent of cardiac work response, after reaching a high level, was maintained In others maximum cardiac performance had been passed at the time of these studies For example in dog 7 after induction of the fistula the cardiac index dropped from 11 liters on the 385th day to 6.7 liters on the 425th day During the interim the dog developed massive edema of the extremities, ascites dyspnea and increased body weight

Although following digitalization the dogs with fistulas varied somewhat in their response the average changes show a slight decrease in the cardiac index cardiac work index and heart rate, with slight increases in mean arterial pressure and total peripheral resistance. The effect of the Lanatoside C in these animals does not correlate with the loads under which the heart was working or with the height of the end-diastolic pressure in the left ventricle. For example, following Lanatoside C the cardiac work index of dog 2 increased even though the cardiac work index was initially only 3,390 gm.M per M^2 per min with a left ventricular end-diastolic pressure of 15 mm Hg while in dog 7 the cardiac work index fell after the drug even though this dog had a high cardiac work level (10 580 gm M per M^2 per min) and a high left ventricular end-diastolic pressure (26 mm. Hg) The effect of the drug on these dogs appears independent of the hemodynamic state of the circulation or of the work load on the heart.

Table III presents the data from five dogs with fistulas that were maintained on digitoxin from 1 to 4 days after initial digitalization with Lanatoside C. Their hemodynamic status was not improved

TABLE IV

*Effect of Lanatoside C in normal dogs**

Dog No	LVEDP		CI		CWI		SVI		SWI		MAP		TPR		HR	
	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
1	9	9	1 8	1 6	1,870	2,000	36	34	36	44	86	103	8 0	11 0	49	46
2	8	8	1 9	2 4	2,500	3,890	29	29	37	47	103	128	9 3	9 3	68	83
3	17	11	2 9	2 1	3,290	2,370	52	48	59	55	100	95	6 4	8 6	56	43
4	7	5	1 7	1 4	1,735	1,440	29	27	29	27	81	79	9 2	10 9	59	53
5	12	12	3 0	2 8	3,080	3,890	40	48	42	65	88	114	5 1	7 0	74	59
6	15	12	2 6	2 0	2,370	1,970	26	24	24	24	83	86	6 2	8 4	101	81
7a	7	7	2 6	2 4	3,610	2,860	43	45	60	53	109	95	8 2	7 5	60	54
b		7		2 4		3,650		40		61		119		9 7		60
Aver	10	9	2 4	2 1	2,640	2,760	37	37	41	47	93	102	7 5	9 0	67	60

* Animals 1, 2, 3, 4, 5, and 6 anesthetized Animal 7 trained, unanesthetized, 7a and b data obtained 30 and 60 minutes, respectively, after digitalization Abbreviations as in Table II

Actually, four of the dogs showed a considerable reduction in cardiac work

The influence of Lanatoside C upon the circulation was assessed in seven dogs without aortic-caval fistulas (Table IV) Six were anesthetized and one was trained and unanesthetized The data in Table IV indicate that the performance of the left ventricle was not greatly altered by Lanatoside C The average changes following rapid digitalization are very similar to those seen in the animals with fistulas A slight decrease in cardiac index and heart rate with an increase in mean arterial pressure and peripheral resistance was noted

DISCUSSION

The results of this study are in agreement with previous reports in the literature which indicate that in the normal heart digitalis, when effective, generally decreases cardiac output and heart rate and increases mean arterial pressure (7, 8)

In the animals with fistulas it is difficult to be certain that heart failure was always present since the direct effects of the fistula would be to produce hind-limb edema, increase venous pressure, and perhaps cause ascites as well However, there were other physical signs and physiological measurements which indicated that these animals were at or beyond their maximum cardiac work level Some animals developed edema of the fore-limbs as well as the hind-limbs, and a number of the dogs died of pulmonary edema which is highly indicative of cardiac failure under these circumstances The maximum cardiac work levels in these preparations were similar to those obtained in normal dogs with massive infusion of blood or saline (1) The left ventricular end-diastolic pressure was consistently elevated in the fistula group, the lowest value being 13 mm Hg with an average value of 24 mm Hg, whereas, the corresponding value for the normal animals was 10 7 mm Hg Furthermore, the infusion of whole blood or saline into these animals led to an additional large increase in left ventricular end-diastolic pressure, and an early decline in cardiac work and output (1) This evidence indicates that these dogs were working at or very near the limit of their cardiac performance

Acute digitalization with Lanatoside C failed to improve the cardiac work response in animals with aortic-caval fistulas The cardiac index did increase in four of the twelve dogs, but the increase was only minimal and one of the animals had shown a similar increase following digitalization before the aortic-caval fistula had been constructed Four of the animals were maintained on digitalis with no improvement, confirmatory evidence that digitalization did not improve cardiac performance These data do not explain the lack of response to digitalis in these animals, but they do indicate that there is an undefined difference between these animals and, for example, animals with cardiac failure produced by pulmonary artery constriction (9)

SUMMARY

Dogs were prepared with a chronic aortic-caval fistula At the time of study, performance of the left ventricle was characterized by a high level of

left ventricular end-diastolic pressure cardiac output, stroke volume and stroke work, and in most instances with one or more evidences indicative of cardiac failure such as pulmonary edema, edema of the extremities and ascites After a period varying from one to 14 months, the dogs were digitalized rapidly with Lanatoside C and the hemodynamic effects were observed during the next hour and in some animals after four days of maintenance dosage on digitoxin Measurements of various cardio-dynamic parameters indicated that digitoxin did not alter measurably the cardiac performance.

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THE DISTRIBUTION OF SODIUM AND CHLORIDE AND THE EXTRACELLULAR FLUID VOLUME IN THE RAT[1]

By DONALD B CHEEK, CLARK D WEST, AND CATHERINE CARTER GOLDEN

(From The Children's Hospital Research Foundation and Department of Pediatrics, University of Cincinnati, Cincinnati, O)

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Our attempts to gain information concerning the amount of electrolyte in cell water and the theoretical concentration of intracellular cations necessarily revolve around our ability to partition total body water into extracellular and intracellular phases The total body potassium, for example, is confined almost exclusively to the intracellular phase, and if the intracellular volume of the body could be defined, the net intracellular potassium concentration could be readily calculated as

$$\frac{\text{Total body K} - \text{Extracellular K}}{\text{Intracellular fluid volume}}$$

The most rational interpretation of extracellular fluid volume would seem to be that defined originally by Manery, Danielson, and Hastings (1), by Manery and Hastings (2) and later by Nichols, Nichols, Weil, and Wallace (3) These authors divide the extracellular fluid into the following components the plasma water, the interstitial water, and the connective tissue water The interstitial water is considered that volume of fluid which rapidly equilibrates with substances such as inulin Connective tissue water is the volume of water which is principally associated with collagen and elastin, and with which substances such as inulin equilibrate slowly The study of Nichols, Nichols, Weil, and Wallace (3) would indicate that the sum of these three phases of the extracellular fluid is defined reasonably well by the chloride space if correction of the chloride space is made for intracellular chloride, and for the slightly greater concentration of chloride in connective tissue water than is present in an ultrafiltrate of serum

The major aim of the present study is to test further the validity of the chloride space, corrected for the factors described above, as a measure of the extracellular volume by demonstrating an identity between it and the space calculated from the sodium content of extracellular fluid Extracellular fluid volume has been calculated from sodium by the expression (extracellular sodium)/$(Na)_{ef}$ where extracellular sodium is the difference between total body sodium and the sodium which is not in the extracellular fluid (*i e*, that in bone, cells, and within the lumen of the gastrointestinal tract) and $(Na)_{ef}$ is the sodium concentration of the serum ultrafiltrate

Values for total body chloride and sodium on which the calculations depend have been obtained by carcass analysis of a large number of normal rats (4, 5) Values for bone sodium have been calculated from the Na/Ca ratio for bone and from total body calcium Values for cell sodium in muscle have been taken from data in the literature (6) calculated on the assumption that chloride in muscle occupies the same space as inulin and an exclusively extracellular position (7) Values for sodium in red cells were also calculated from the data of other investigators (8, 9) while those in visceral cells have been assessed by comparison of the volumes of distribution of inulin and sodium in the viscera following a constant infusion of inulin according to the technique described by Cotlove (7)

The good agreement between the sodium and chloride spaces calculated by correcting the value for total body electrolyte in this manner lends credence to the view that a value closely approximating the true extracellular volume of the rat has been determined On this assumption the data obtained by carcass analysis have been used for further calculation of the intracellular volume and the theoretical concentrations of electrolyte within the cells

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METHODS AND CALCULATIONS

The animals used in the study were male Wistar rats of the Hamilton Farms strain. For convenience and simplicity a rat with a fat free dry solid content of 50 grams has been taken as a representative animal. Assuming a normal body fat content, such an animal would weigh approximately 220 grams.

Total body electrolyte and water The carcass electrolyte and water content of the hypothetical 220-gm. rat was determined from regression equations calculated from data obtained by carcass analysis of 30 to 40 rats of normal health and vigor The weight range of the animals was from 70 to 410 gm. with the greatest number falling around 220 grams (4 5) The animals had been under observation at least two weeks prior to sacrifice and for ten days had been on a low residue diet of composition previously described (4) The regressions of total body electrolyte, water and nitrogen on fat free dry solid are given in Table I.

The methods of carcass analysis, with the exception of that for potassium have been given previously (4 5) In previous studies potassium was determined on an acid extract of ashed carcass by flame photometry while in the present study potassium in the ash has also been determined with chloroplatinic acid by the method of Consolazio and Talbott (10) modified to determine the salt K_2PtCl_6 gravimetrically instead of by iodometric titration. The method is to be published later in detail (11) The flame photometric method, employed on 47 animals yielded a regression equation with a lower slope (0.283) a higher intercept (+0.36) and a greater scatter of the data (standard error of the estimate, 1 08 mEq) than the gravimetric procedure. For animals of a fat free dry solid content of 50 gm. with which the present paper is concerned, the two equations gave almost identical values. By the gravimetric procedure, carcass potassium would be 14.38 mEq as compared with 14.51 by flame photometry

Estimation of bone sodium Bone sodium was estimated in five healthy male rats of 210 to 230-gm. weight. These animals were of the same strain and prepared in the same manner as those used for carcass analysis. After sacrifice and exsanguination, the carcass was dried at 100 to 105 C as previously described. After drying the larger bones could be easily removed by pulling away the dried tissues and cutting free adherent cartilage. The bones were broken into small pieces, the fat extracted in a Soxhlet apparatus and the pieces further titrated to fine particles in a mortar A portion of the particles were then ashed in a platinum crucible and the ash dissolved in 5 0 ml. of weak HCl Separate aliquots were taken for calcium determination by permanganate titration and for sodium determination by the gravimetric method of Butler and Tuthill (12) A separate aliquot of the bone particles was subjected to alkaline ashing and analyzed for chloride by the microdiffusion technique. The methods are identical to those used for carcass and are described in greater detail in previous papers (4 5) The difficulties inherent in the flame photometric determination of bone sodium through interference by calcium (13) are obviated by use of the gravimetric procedure.

The sodium incorporated in bone salt was determined by subtracting from total bone sodium the amount of sodium calculated as present in the chloride space of bone. The total bone salt sodium of the carcass was calculated as total bone salt sodium

$$= \frac{\text{bone salt sodium (mEq./100 gm. FFDS)}}{\text{bone calcium (mEq./100 gm. FFDS)}} \times \text{total body calcium (mEq.)}$$

Determination of total body collagen and of connective tissue chloride Determinations of carcass collagen were performed on nine rats weighing approximately 220 gm., prepared before sacrifice in the same manner as those used for carcass analysis. The method of collagen estimation was that of Spencer Morgulis, and Wilder (14) A homogeneous aliquot of powdered, fat free, dry carcass was autoclaved to convert collagen to gelatin, the gelatin was extracted with hot water and finally precipitated with tannic acid.

The chloride concentration of connective tissue water was determined by analysis of the achilles tendon of a normal rat and of a normal female mongrel dog After exposure of the tendon dissection was carried out rapidly and each piece removed was immediately placed in a

TABLE I

Body composition in the normal rat (wt range 70 to 410 grams) Regressions of total body electrolyte nitrogen and water on fat free dry solid

Regression equations	Standard error of estimate	Number of rats	Values for 220-gm rat (FFDS = 50 gm)
Na_t (mEq) = 0 193 FFDS + 0 78	± 0 64 mEq	36	10 43 mEq
Cl_t (mEq) = 0 131 FFDS + 1 04	± 0 41 mEq	35	6 73 mEq
K_t (mEq) = 0.323 FFDS − 1 77	± 0 75 mEq	29	14.38 mEq
Mg_t (mEq) = 0 1014 FFDS + 0 594	± 0 32 mEq	32	5 66 mEq
Ca_t (mEq) = 2.200 FFDS − 1 74	±11.50 mEq	35	111 70 mEq
P_t (mM) = 0 849 FFDS + 0.300	± 3.20 mM	30	42 75 mM
N_t (grams) = 0 131 FFDS − 0 065	± 0 200 gm.	40	6 49 gm
H_2O_t (ml) = 2 725 FFDS + 13 19	± 3 70 ml.	36	149 40 ml
Average fat content 9 6% body wt. Range 15 3 to 3 3%		36	

te tared stoppered weighing bottle to minimize ation of water After weighing, the tendon was to constant weight at 100 to 105°C, reweighed, l in a mortar and fat extracted. Analyses for le, collagen and total nitrogen were carried out as cass (4, 5, 14)

rmination of extracellular sodium and chloride of | Using inulin as a reference substance, sodium iloride were partitioned between extra and intra-r compartments in the lungs, trachea, esophagus, :h, intestines and testes by the method described by e for muscle (7) The data were obtained on 11 rats whose weights ranged from 290 to 310 gm. nimals were placed on a low residue diet (4) four efore experimentation and fasted for 24 hours be-acrifice. Under ether anesthesia either a poly-1e catheter or a 22 gauge needle was placed in a in for inulin infusion and the animal confined in paratus described by Kellogg, Burack, and Issel- (15) Following priming with an amount of calculated as sufficient to raise the plasma inulin tration to 300 mg per cent, an infusion of inulin aintained at a constant rate using an infusion ma- Both priming and maintenance solutions of inu-ntained 7 5 gm. of inulin and 0 64 gm of sodium le per 100 ml After 6 hours of infusion in 7 ani-nd after 24 hours in 4 animals, ether anesthesia was applied and the animal exsanguinated by aortic re. The infusion was stopped at the moment of puncture. The lungs, gastrointestinal tract and were then quickly removed and placed in a tared and weighed. Forty milliliters of water were then and the tissues homogenized in a micro Waring r The homogenate was placed in a boiling wa-th for 30 minutes and then filtered. While in the bath, the tubes were covered with aluminum foil ace evaporation Serum and filtrate were analyzed dium by flame photometry and for inulin by the d of Schreiner (16) after preliminary hydrolysis alkali as described by Ross and Mokotoff (17) de in the tissue filtrate was determined by the diffusion technique of Conway (4, 18) and serum le by the method of Van Slyke (19) In early ments the liver was analyzed separately, but the re not included in the presentation because, after irs of infusion, the inulin space was found to be than either the sodium or chloride space.

inoid blanks were determined on the serum and : of the visceral organs of 13 rats which received lin. These animals had been on the same diet as perimental animals for four days before sacrifice sted for 24 hours before sacrifice. The inulinoid for serum after alkali hydrolysis were found to be ible and were disregarded. Blank values for the : of viscera ranged from 2 4 to 4.7 mg per cent. verage value was 3 44 and the standard deviation mg per cent. Inulin concentrations in the viscera s of rats infused with inulin ranged from 25 4 to ig per cent with an average value of 38 0 mg per cent. Serum inulin levels averaged 290 with a range from 209 to 428 mg per cent. The total inulin and electrolyte content of the tissue was calculated as the product of viscera filtrate concentration times the volume of added water plus tissue water Tissue water was taken as 81 per cent of wet weight (20)

The sodium and chloride spaces in viscera were corrected for the quantities of sodium or chloride present in the lumen of the gastrointestinal tract. The amount of the correction was determined by analysis of the luminal contents of five rats which had been on a low residue diet for four days and fasted for 24 hours before sacrifice. No infusion was given After anesthetizing with ether, the lower end of the gastrointestinal tract was clamped, the gut dissected free and placed in a beaker A rubber catheter was then inserted in the cardia of the stomach and 50 ml of 5 per cent glucose in water rapidly injected so that the full length of the gut was distended. The clamp was then removed and the fluid gently pressed out through several incisions made in the gut wall The whole procedure required only a few minutes The volume of fluid was then measured and, after filtering, analyzed for sodium and chloride. The volume of fluid recovered averaged 49 6 ml.

Estimation of gastrointestinal fluid volume The values for total body water determined by carcass analysis were corrected for the water contained within the gastrointestinal tract. The volume of fluid within the gut lumen was determined in six rats weighing approximately 220 grams The animals were prepared in a manner identical to those subjected to carcass analysis, a low residue diet was fed for five days with free access to water They were then sacrificed after a four-hour fast at the same time of day as the animals used for carcass analysis The gastrointestinal tract was dissected free, placed on a board and opened longitudinally with scissors The gut surface was then gently swabbed with gauze which had been previously dried by heating at 100° C The gauze was immediately placed in a weighing bottle, covered and weighed. The bottles were then uncovered, placed in an oven at 100° C for 24 hours and re-weighed. The water content of the gut in milliliters was taken as the weight difference of the gauze in grams

Calculation of sodium, chloride, and inulin spaces The volumes of distribution of sodium and chloride were calculated by dividing the amount of the ion present by the concentration in a serum ultrafiltrate. To determine serum ultrafiltrate concentration, the serum concentration was corrected for serum water and Donnan equilibrium as follows

$$(\mathrm{Na})_{st} = \frac{(\mathrm{Na})_s}{0\ 93} \times 0\ 95$$

and

$$(\mathrm{Cl})_{st} = \frac{(\mathrm{Cl})_s}{0\ 93} \times \frac{1}{0\ 95}$$

in which $(\mathrm{Na})_{st}$ and $(\mathrm{Cl})_{st}$ are the amounts of sodium and chloride per liter of serum ultrafiltrate, $(\mathrm{Na})_s$ and $(\mathrm{Cl})_s$ the amounts per liter of serum, and 0 95 the Don-

nan factor Serum water was taken as 930 ml. per liter of serum and is represented by the factor 0.93

Serum ultrafiltrate concentrations of inulin were calculated as $(In)_s/0.93$ where 0.93 is again the serum water correction.

RESULTS

Calculation of extracellular volume from extracellular chloride

The total body chloride of a 220-gram rat and the corrections necessary for the estimation of that portion of total chloride which is in extracellular fluid and at a concentration equal to that in a serum ultrafiltrate are given in Table II A description of the calculation of these corrections follows.

Correction for connective tissue chloride To correct total body chloride for the chloride in connective tissue in excess of that predicted from the chloride concentration of the serum ultrafiltrate, analyses of dog and rat tendon for water chloride collagen and total nitrogen were carried out. The data are given in Table III In both species the actual chloride content of tendon was 0.76 mEq per 100 gm of FFDS greater than would be predicted from the tendon water content and the serum ultrafiltrate chloride concentration In terms

TABLE II

Calculation of extracellular fluid volume from extracellular chloride in a 220-gram rat (50 gram FFDS)

	mEq	Percent of Total
Chloride Outside Extracellular Fluid		
Chloride of connective tissue in excess of predicted (from determination of "excess" chloride in rat and dog tendon and from rat total body collagen)	0.10	1.5
Erythrocyte chloride (from chloride concentration of red cell water (8) blood volume and hematocrit (9) and red cell water content)	0.41	6.1
Chloride intracellular in muscle (7)	0	0
Chloride intracellular in liver (from difference between sodium and chloride space of liver)	0.06	0.9
Chloride within lumen of gut (determined by flushing of gut)	0.16	2.4
Chloride intracellular in viscera exclusive of liver brain spleen and kidneys (from comparison of sodium chloride and inulin spaces)	0.12	1.8
Total	0.85	12.7
Total Body Chloride	6.73	
Chloride in Extracellular Fluid	5.88	87.3

Serum ultrafiltrate chloride concentration 125 mEq/L.

Chloride space $\frac{5.88}{.125}$ 47.0 ml.

TABLE III

Chloride and water content of tendon in dog and rat Calculation of "excess" chloride in tendon

	(1) Tendon water mL/100 gm FFDS	(2) $(Cl)_{uf}$* mEq./L.	(3) Tendon Cl predicted $(1)\times\frac{(2)}{1,000}$ mEq/100 gm FFDS	(4) Tendon Cl found mEq./100 gm FFDS	(5) "Excess" chloride (4)−(3) mEq/100 gm FFDS
Dog	128	127	16.26	17.02	0.76
Rat	140	125	17.50	18.26	0.76

* $(Cl)_{uf}$ = Chloride concentration of serum ultrafiltrate

of tendon water content, tendon chloride amounted to 133 mEq per L in the dog and 131 mEq per L. in the rat in both species the values were 6 mEq per L greater than the concentration of the serum ultrafiltrate

Taking the excess chloride in connective tissue as 0.76 mEq per 100 gm of FFDS the excess chloride associated with collagen in the whole rat may be calculated as 0.76/100 × total body collagen (grams) Total body collagen in nine rats of approximately 220 grams body weight averaged 11.9 ± 0.48 (SD) grams The excess chloride associated with connective tissue then becomes 0.10 mEq The calculation assumes that all body collagen has the same affinity for chloride as does achilles tendon No correction has been made for the fact that achilles tendon is not pure collagen by analysis only 91 per cent of the total nitrogen of the tendon could be accounted for as collagen nitrogen

Erythrocyte chloride Calculation of the intracellular chloride of erythrocytes was made from the data of Bernstein (8) which give the chloride concentration of the rat red cell water and from the data of Wang and Hegsted (9) which define the blood volume and hematocrit According to the latter authors the blood volume of a 220-gram rat closely approximates 7 per cent of the body weight and the hematocrit averages 45 Red cell water was taken as 72 grams per 100 ml and the chloride concentration of red cell water as 72 mEq per L. (8) Using these values the chloride intracellular in erythrocytes may be calculated as 1.86 mEq per Kg of body weight In a 220-gram rat the value would be 0.41 mEq

Chloride intracellular in muscle and liver Based on the studies of Cotlove (7) comparing the distribution volumes of inulin and chloride in

muscle after prolonged inulin infusion, the assumption has been made that chloride is not present in muscle cells A similar assumption cannot be made for the liver In this organ the volume of distribution of chloride exceeds that of sodium so that an intracellular position of chloride must be presumed For lack of a better method, intracellular chloride in liver has been calculated on the assumption that the true extracellular volume of this organ is measured by the volume of distribution of sodium In seven rats, the chloride space of liver exceeded the sodium space by 0 5 to 0 95 ml corresponding to 0 066 to 0 120 mEq of chloride The average value in the 300-gram rat of 0 088 mEq of intracellular chloride becomes 0 060 mEq for a 220-gram rat

Chloride within the lumen of the gastrointestinal tract The chloride in the "transcellular" fluid within the gut was determined by flushing the intestinal tract with isotonic glucose solution In five rats weighing approximately 300 grams, the chloride recovered averaged 0 22 mEq and ranged from 0 14 to 0 28 mEq Corrected to a body weight of 220 grams, the average value becomes 0 16 mEq Several sources of error in this determination should be mentioned Although the flushing procedure was carried out as rapidly as possible, the possibility cannot be excluded that some chloride diffused from the extracellular fluid through gut mucosa or serosa or through the incisions made in the gut wall Also, the data were for use primarily in correcting the chloride space of viscera and to compare this space with the inulin space, as will be described below Consequently the animals were not prepared in exactly the same manner as those used for carcass analysis Food was withheld from those used for carcass analysis for only four hours while those used for the flushing procedure were starved for 24 hours Although both groups had been on the same low residue diet, the differences in food intake might have produced differences in intraluminal gut chloride It seems possible that these two sources of error, the one tending to give a falsely high value and the other a value falsely low, could balance out

Chloride in cells of viscera exclusive of liver, brain, spleen and kidneys Although chloride has been described as being intracellularly located in the pylorus, testes and lungs (2) the amounts present and their significance have not been clearly defined The problem has been approached in the present study by determining the inulin, chloride and sodium spaces of gut, lung and testes after prolonged infusion of inulin The data for these volumes are shown in Table IV In this table the chloride and sodium spaces have been corrected for the amounts of these ions determined by

TABLE IV

Inulin and corrected chloride and sodium spaces of viscera after 6 and 24 hours of inulin infusion

Rat no	Wt. of viscera *gm*	Inulin space *ml*	Inulin sp / Viscera wt.	Corrected chloride space *ml*	Corrected sodium space *ml*	Inulin sp / Cl space	Inulin sp / Na space
			After 6 hours of inulin infusion				
2	21 4	5 35	25	8 55	5 23	0 626	1 02
3	21 0	6 88	33	6 21	4 95	1 11	1 39
4	23 5	7 23	31	7 43	6 16	0 974	1 17
7	22 1	6 89	31	8 62	7 92	0 80	0 87
10	20 5	6 56	32	8 33	6 73	0 788	0 97
11	21 1	5 42	26	6 57	5 42	8 826	1 00
12	21 1	6 26	30	7 43	5 91	0 842	1 06
Averages			29	7 59	6 04	0 852	1 07
			After 24 hours of inulin infusion				
5	22 6	6 11	27	7 38	6 89	0 828	0 89
9	19 8	6 33	32	6 06	5 78	1 04	1 10
13	23 7	9 93	42	9 23	6 30	1 076	1 57
14	25 7	8 53	33	7 48	6 84	1 19	1 25
Averages			33	7 54	6 45	1 021	1 20
Averages (Both groups)				7.57	6 19		

the flushing experiments (see above) to be present in the gut lumen. This correction amounted to 0.22 mEq of chloride and 0 43 mEq of sodium for the 300-gram rats used.

At the outset it should be emphasized that use of the inulin space measurement for determining the amounts of sodium and chloride within the cells is hazardous Although the measurement of inulin space by tissue analysis for inulin precludes errors in space measurement due to metabolism of inulin it does not preclude error resulting from sequestration of inulin by macrophages as recently suggested by White and Rolf (21) These authors found that tissues rich in macrophages gave impossibly high inulin space values With this source of error in mind, the present data can probably be interpreted as reliably by simple comparison of sodium and chloride spaces as by resorting to inulin space measurement. It can be seen that in every specimen the corrected chloride space exceeded the corrected sodium space the difference averaging 1 38 ml Assuming no sodium to be present in the cells and the sodium space to equal extracellular volume the greater chloride space would represent chloride within the cells The volume of 1.38 ml is equivalent to approximately 0 17 mEq of chloride or corrected to a 220-gram rat, 0 12 mEq In agreement with this interpretation is the inulin space at 6 hours Inulin space at this time was in fair agreement with the sodium space but was smaller than the chloride space by 15 per cent. Taking the inulin space at 6 hours as approximating extracellular fluid volume, an intracellular chloride content similar to that defined above would be calculated. By this interpretation the further expansion of the inulin space at 24 hours to equality with the chloride space and to 33 per cent of the wet weight of the tissue would be ascribed to macrophage sequestration of inulin

Disregarding the possibility of macrophage sequestration and using only the 24-hour values the data could be alternatively interpreted as indicating that at 24 hours complete penetration of extracellular fluid by inulin had occurred The identity of inulin and chloride spaces at this time would then indicate no chloride to be intracellular in viscera, a conclusion contrary to that of previous workers Since this interpretation does not account for the smaller sodium space, the calculation based on the sodium space, indicating that 0 12 mEq of chloride is present intracellularly in viscera, seems more reasonable.

Estimation of chloride space As may be seen in Table II the chloride which must be considered as outside the extracellular fluid amounts in a 220-gram rat, to 0.85 mEq or 12.7 per cent of body chloride. Of this non-extracellular chloride, the greatest amounts are found in the red cells and in the transcellular fluid in the lumen of the gastrointestinal tract. Because of the technical difficulties in assessing the position of chloride in brain, spleen and kidneys these organs have not been included in the calculations Their omission would appear to be of little consequence. In the rat the combined weight of these organs amounts to only 1 5 per cent of body weight and from the data of Manery and Hastings (2) it can be calculated that their chloride content amounts to only 2.2 per cent of total body chloride. Thus even if a relatively large fraction of the chloride of these organs were intracellular it would constitute only a small fraction of the total body chloride

For the 220-gram rat the chloride in extracellular fluid amounts to 4.88 mEq (Table II) The value for serum chloride concentration used in calculating the chloride space was determined by analysis of the sera of 37 normal rats The average was 109 5 mEq per L with a range from 105.6 to 113 6 Correcting the average value for serum water and for the Donnan factor gives a value of 124 mEq per L. for the serum ultrafiltrate concentration and a chloride space of 47 4 ml

Extracellular space calculated from extracellular sodium

Extracellular sodium has been calculated as a means of verifying the accuracy of the extracellular volume predicted from the distribution of chloride. The calculated amounts of sodium in cells and bone in a 220-gram rat are shown in Table V and the calculations are described below

Bone sodium In five rats weighing approximately 220 grams the bone salt sodium calcium ratio was found to average 0 0200 with a range of 0 0187 to 0 0205 (Table VI) Total bone calcium in a 220-gram rat, calculated from the regression equation shown in Table I amounts to 111.7 mEq Multiplying the bone salt sodium

TABLE V

Calculation of sodium in extracellular fluid and extracellular fluid volume of a 220-gram rat (50 gram FFDS)

	mEq	Percent of Total
Sodium Outside Extracellular Fluid		
Bone salt sodium (from Na:Ca ratio for bone salt and total body Ca)	2 21	21 2
Sodium intracellular in muscle (from rat muscle analyses (6) and muscle mass (25))	0.60	5 8
Erythrocyte sodium (from sodium concentration of red cell water (8) blood volume and hematocrit (9) and red cell water content)	0 14	1 3
Sodium within lumen of gut (determined by flushing of gut)	0 32	3 1
Sodium in cells of viscera (from comparison of sodium and inulin spaces after 6 hrs of inulin infusion)	0	0
Total	3 27	31 4
Total Body Sodium	10 43	
Sodium in Extracellular Fluid	7.16	68 6

Serum ultrafiltrate sodium concentration, 149.6 mEq/L

$$\text{Sodium space} = \frac{7.16}{.1496} = 47\ 9 \text{ ml}$$

alcium ratio by total body calcium gave an aver-ge value of 2 21 mEq for total bone salt sodium

The accuracy of the estimate of the bone salt odium is of importance in the assessment of elec-rolyte distribution because of the large fraction f body sodium present in bone Possible sources f error lie in the method of estimation of the odium calcium ratio and in the assumption in-erent in the calculation that the sodium cal-um ratio of all bones is the same as that of the pecimens of bone taken for analysis [2] The gen-ral agreement of the sodium calcium ratio of 0200 obtained in the present study with that of 0206 obtained by Bergstrom (22) by flame hotometric determination of sodium suggests the bsence of gross errors in analysis The validity f the assumption that all bones have the same sodium calcium ratio is more difficult to assess From fragmentary data, however, the assumption in the main appears to be correct Agna and Knowles (23) have found in nine human subjects that rib and iliac crest are almost identical in their sodium calcium ratios On the other hand, the skull contained an average of 7 per cent more sodium per unit of calcium than did rib or ilium Appendicular skeleton was not analyzed [3]

Muscle cell sodium Based on the data for muscle analyses of 13 rats by Cotlove, Holliday, Schwartz, and Wallace (6) the sodium intracellular in muscle was taken as 0 6 mEq per 100 grams of fat free tissue The muscle mass of the rat was taken from the data of Caster, Poncelet, Simon, and Armstrong (25) These authors were able to measure not only the muscle mass that was easily dissected from the skeleton but also the fraction adhering after dissection The latter fraction was determined either from the composition of the ash or from the determination of actomyosin The muscle mass of 320 to 350-gram rats of good nutritional status was found by these authors to average 45 5 per cent of body weight In a 220-gram rat the muscle mass would closely approximate 100 grams and would contain 0 6 mEq per L sodium in the intracellular space

Erythrocyte sodium After Bernstein (8), the sodium concentration of red cell water is taken as 28 mEq per L The value of 0 14 mEq for total red cell sodium was calculated in the manner described for erythrocyte chloride

Sodium in lumen of the gastrointestinal tract The quantity of sodium in the transcellular fluid in the gastrointestinal tract was determined as for chloride by the flushing procedure The sodium recovered averaged 0 43 mEq with the range of 0 35 to 0 50 The amount in the 220-gram rat

[2] Knowing the weights and calcium content of the one samples taken for analysis and the total body cal-um, the fraction of total skeleton represented by the bone samples may be calculated. The bone sample weights in terms of fat free dry solid averaged 1 45 grams and their calcium content averaged 10 7 mEq per gram FFDS With an average total body calcium content in a 220-gram rat of 111.7 mEq the total skeleton as FFDS may be calculated from the above as weighing 10 4 grams The bone samples then represented on the average 14 per cent of the total skeleton.

[3] If in the rat the sodium calcium ratio of the skull were greater than the rest of the skeleton by 5 per cent as appears to be the case in the human, the error resulting from neglect of this discrepancy in the present calculations would be minimal. The dried skull of a 220 gram rat weighs 1 42 grams (24) Assuming the calcium content of dried skull to be 10 7 mEq per gram and a 5 per cent greater sodium calcium ratio for skull, the bone sodium would be greater than that found in the present calculation by 0 015 mEq This amount represents 0 02 per cent of the total body sodium.

TABLE VI

Average values and range for bone calcium sodium and chloride content and the bone salt sodium bone calcium ratio

No. of rats	Sodium *mEq/100 gm. FFDS*	Calcium *mEq/100 gm. FFDS*	Chloride *mEq/100 gm. FFDS*	Chloride space *ml./100 gm. FFDS*	Sodium in chloride space *mEq/100 gm. FFDS*	Bone salt sodium / Bone calcium
5	29 7 32 8–26 7	1,074 4 1 032–1 109	6 83 8 65–5.23	55 1	8 23	0 0200 0 0187–0 0205

would be 0 32 mEq or slightly more than 3 per cent of total body sodium.

Sodium intracellular in viscera As mentioned above the liver was considered to contain no intracellular sodium the volume of distribution of this ion was considered as a measure of the extracellular space In the rest of the viscera studied (lungs, gastrointestinal tract and testes) the comparison of the inulin with the sodium space was used to assess the amount of sodium located intracellularly For the comparison the sodium space was corrected for the amount of sodium found by the flushing procedure to be present in the gut lumen The correction amounted to 0 43 mEq As may be seen in Table IV the inulin space approximates the corrected sodium space after six hours of inulin infusion. The data have been interpreted to indicate that no sodium is present within the cells of the viscera.

Estimation of extracellular sodium space Summing up these corrections in Table V it is seen that the sodium outside the extracellular space in a 220-gram rat would be 3.27 mEq or 31 4 per cent of total body sodium

For calculation of the extracellular sodium space the value for extracellular sodium (7 16 mEq) has been divided by the average sodium concentration in a serum ultrafiltrate Serum sodium concentration in 15 normal rats averaged 146 7 mEq per L and the calculated serum ultrafiltrate concentration 149 6 mEq per L. The resultant extracellular sodium space of 47.9 ml is in good agreement with the extracellular chloride space of 47 4 ml

Partitioning of body water, sodium and potassium Total body water in a 220-gram rat averaged 149 4 ml. (Table I) Assuming extracellular volume as 48 ml the extracellular phase would constitute 32 per cent of total body water or 21.8 per cent of body weight

The partitioning of sodium in the body and the net concentrations of body sodium potassium and magnesium in intracellular water are shown in Figure 1 For the calculation of intracellular volume, the volume of fluid within the gut was determined on six rats of 220-gram weight The average value was 3 6 ± 0 42 (SD) ml Subtracting this value and that of 48 ml for extracellular volume from total body water gives 97.8 ml for the volume of intracellular water Extracellular potassium in a 220-gram rat was calculated as 0 19 mEq The net concentration of potassium in cell water calculated as shown in Figure 1 would be 146 mEq per liter Sodium concentration in cell water, similarly calculated, would be 7 4 mEq per liter

For the calculation of the magnesium concentration of cell water the data of Duckworth Godden and Warnock (26) have been used These authors found the calcium magnesium ratio of rat bone to be 41.8. From this ratio and from total body calcium it may be calculated that 2 68 mEq of magnesium are in the bones of a 220-gram

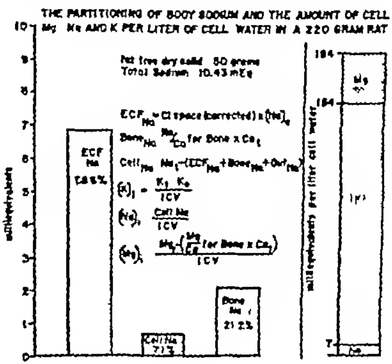

FIG. 1

rat, leaving 3 mEq in the soft tissues Neglecting extracellular magnesium the cell water concentration of magnesium would be 30 mEq per liter This value agrees well with that of 33 mEq per liter found by other investigators (6) for the magnesium concentration of muscle cell water Similarly, from the calcium phosphorus ratio for bone (26) and from total body calcium it can be calculated that 35 3 mMols of phosphorus (or 83 per cent of total body phosphorus) are present in bone

DISCUSSION

For the precise interpretation of change in intracellular electrolyte during metabolic studies and for the proper partitioning of electrolyte in the body it is desirable that extracellular and intracellular volumes be defined The present study has approached the problem of volume measurement by determining extracellular volume from extracellular chloride An attempt has been made to verify the result by demonstrating that the sodium content of this extracellular volume corresponds in amount with that calculated from total body sodium minus the amounts present in bone and in cells The good agreement of these independent approaches strongly suggests that extracellular volume has been correctly measured

In spite of the good agreement, it should in all fairness be pointed out that errors could still exist which, if present on both the sodium and chloride sides of the balance, would cancel out Possible errors in the assessment of bone sodium and of intraluminal gut sodium and chloride have already been mentioned The exact quantitation of the amounts of sodium and chloride in visceral cells is likewise a difficult problem and may be a source of error If the assumption that no sodium is present in the cells of the viscera were incorrect, the error would balance out and not be detected by the approach used Recourse to inulin as a marker to divide the intra- from the extracellular phase has limitations The technique of constant infusion and tissue analysis excludes the error resulting from metabolism of inulin (27) but does not exclude error from accumulation of inulin in macrophages Although inulin does not penetrate most cells under ordinary circumstances, unexpectedly high values for inulin space are obtained in anuric patients (28) and in nephrectomized rats (21) Under conditions of nephrectomy, many substances normally extracellular in distribution predict volumes which are unreasonably high (29) and the possibility cannot be excluded that under these conditions ions or molecules penetrate the cellular phase White and Rolf (21) using the nephrectomized rat and tissue analysis for inulin obtained a progressively large inulin space which, after 72 hours, predicted volumes exceeding total body water Such findings may be related in some way to the removal of renal tissue, for the inulin space of rat muscle was significantly higher than that demonstrated by Cotlove (7) in animals with intact kidneys constantly infused with inulin In White and Rolf's study (21), however, the anomaly was greatest in tissues rich in macrophages, such as liver, and the authors suggest that sequestration of inulin can occur in macrophage cells In the non-nephrectomized animals in the present study it was found that in liver, the inulin space greatly exceeded both the sodium and chloride spaces after 24 hours of infusion Hence, absence of renal tissue appears not to be the sole factor responsible for the large inulin spaces of nephrectomized animals Whether in the other viscera studied, macrophage sequestration of inulin accounted for the increase in inulin space relative to sodium space as the inulin infusion was prolonged to 24 hours (Table IV) cannot be determined This interpretation seems the most plausible, however, when the data are considered from all aspects, and the use of the six-hour inulin space, or the closely similar sodium space, as an approximation of extracellular volume appears to be justified

A further source of error lies in the estimation of the "excess" chloride of connective tissue It should be noted that the calculations assume that all of body collagen has an affinity for "excess" chloride equal to that of the large tendon masses taken for analysis This assumption is probably in the main correct, as pointed out by Manery, Danielson, and Hastings (1), insofar as the connective tissue of muscle, which accounts for the majority of body collagen, is a direct extension of and probably similar in structure to tendon However, the collagen of bone matrix and in visceral organs could well differ from tendon in its affinity for chloride Also the wide differences in the amount of excess chloride in connective tissue

found by various investigators should be borne in mind. Manery Danielson and Hastings (1) found the chloride concentration of connective tissue water in rabbits to average 8 4 mEq per L. greater than the concentration in a serum ultrafiltrate a value in good agreement with that of 6 mEq per L. found in the present study On the other hand another group of workers (30) found the concentration in dogs to be approximately equal to that of a serum ultrafiltrate while more recently a concentration in connective tissue water greater by 56 mEq per L than that of the serum ultrafiltrate has been reported in rats (31) It is possible that some of these differences are attributable to difficulties inherent in the methods of chloride analysis (4) the loss of variable amounts of water at the time of dissection would appear not to be the sole responsible factor

While it is obvious from the above that errors may be present most of them would appear to be of relatively little significance to the present study As it stands the study predicts that 87 per cent of total body chloride is in the extracellular fluid Of the 13 per cent located outside of this compartment more than half is in the erythrocytes and in the gastrointestinal tract Because the amounts in cells and 'excess in connective tissue are so small even gross errors in the quantitation of these fractions would have little effect on the overall assessment For example, doubling the amount of 'excess chloride associated with connective tissue would reduce the present estimate of extracellular chloride by only 1 5 per cent. The conclusion that most of body chloride is in extracellular fluid appears justified The data do not support the contention of other investigators employing differing techniques (32 33) that 30 per cent or more of body chloride resides outside the extracellular fluid

Previously, Manery and Hastings (2) Manery and Haege (34) and Amberson, Nash Mulder, and Binns (35) have produced considerable evidence for the contention that chloride is predominantly extracellular in various tissues At the same time these studies have suggested that intracellular chloride exists in pyloric tissue stomach fundus and testes

It would seem of importance that the exchangeable chloride of the rat measured with bromide over a 3-hour period closely predicts the true carcass chloride (4) A corollary of the findings presented is that the bromide or radio chloride space corrected for red cell bromide or chloride gives a close approximation of all phases of the extracellular fluid volume in the normal subject

The value of 146 mEq per L. obtained in the present study for the potassium concentration of cell water agrees well with the value of 140 mEq per L obtained by Harrison Darrow and Yannet (36) for the dog by carcass analysis using the uncorrected chloride space as the measure of extracellular volume. By indirect methods however, widely diverging values have been obtained Using inulin, D_2O K^{42} and Na^{24} the intracellular potassium and sodium concentration of the dog averaged 115 and 35 mEq per liter of cell water respectively (37) These concentrations are greater for sodium and lower for potassium when comparison is made with the present data Possibly inulin under the conditions of these dog experiments under-estimates the total extracellular volume (38) Moore (39) using thiocyanate as a measure of extracellular volume and D_2O K^{42} and Na^{24} to measure total water exchangeable potassium and sodium, respectively predicted in man an intracellular potassium concentration of 163 mEq per liter of cell water No estimate of transcellular fluid was possible in this study This potassium concentration would seem to be high as a result of the fact that thiocyanate over-estimates the extracellular space (40 41)

Evidence is at hand that intracellular and extracellular osmolar concentrations in the rat are equal (42) There is also some evidence that the major fraction of the magnesium of intracellular fluid is not dissociated (43 44) and is probably bound to protein and phosphate anions (44) so that almost all of the intracellular potassium should be osmotically active However Macallum demonstrated more than 50 years ago (45) that while most of the potassium of the cell is evenly distributed throughout the cytoplasm local points of high concentration can be detected More modern investigations (46) suggest that about 13 per cent of cell potassium is not osmotically active and is present in mitochondria The present study does not allow conclusions regarding the tonicity of cell fluid.

SUMMARY

An attempt has been made to determine the amount of sodium and chloride in the extracellular fluid in the rat by correcting total body sodium and chloride for the amounts of these ions which are outside the extracellular fluid Corrections for chloride included the "excess" chloride of connective tissue calculated from total body collagen and the results of tendon analysis, chloride intracellular in liver calculated from the sodium space of liver, and chloride in the lumen of the gut determined from analysis of gut contents Erythrocyte chloride was calculated from data in the literature The intracellular chloride of the respiratory and gastrointestinal tracts and in the testes was assessed by tissue analysis after the constant infusion of inulin From data of other investigators, it was concluded that no chloride is present intracellularly in muscle

It was found that 87 3 per cent of body chloride can be considered as present in extracellular fluid The chloride in erythrocytes and in the gut lumen, representing 6 1 and 2 4 per cent of total body chloride, respectively, account for the bulk of the non-extracellular chloride

To obtain extracellular sodium, total body sodium was corrected for bone salt sodium, calculated as the product of the Na/Ca ratio for bone and total body calcium, and for sodium in the lumen of the gut, determined from the analysis of gut contents Data in the literature were used for the calculation of erythrocyte sodium and for the intracellular sodium of muscle

For a 220-gram rat, extracellular fluid volume calculated from chloride was found to be 47 4 ml as compared with 47 9 ml calculated from sodium The good agreement between these values suggests that this approach and the corrections used are valid Extracellular volume would thus represent 21 8 per cent of body weight or 32 per cent of body water

From the data for extracellular volume and from other parameters of body composition obtained from carcass analysis and from the literature, the theoretical net concentrations of potassium, sodium and magnesium in cell water have been calculated

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INCORPORATION OF N[15]-L-ASPARTIC ACID INTO THE ABNORMAL SERUM AND URINE PROTEINS OF MULTIPLE MYELOMA (STUDIES OF THE INTER-RELATIONSHIP OF THESE PROTEINS)[1]

By ELLIOTT F OSSERMAN, ADA GRAFF, MARGARET MARSHALL, DOLORES LAWLOR, AND SAMUEL GRAFF

(From the Departments of Medicine and Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N Y, and the Medical Service and Biochemistry Division, Francis Delafield Hospital, New York, N Y)

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In a consideration of the abnormalities of protein metabolism observed in multiple myeloma, a number of fundamental problems remain unsolved To state just two of these, it remains to be determined (a) precisely what relationship the abnormal serum globulins of myeloma bear to normal serum gamma globulin, and (b) what is the nature of the possible inter-relationship between the abnormal serum protein and urinary (Bence-Jones) proteins of myeloma when both are present in a particular case The electrophoretic homogeneity of these abnormal serum and urinary proteins constitutes one of their major physicochemical features Using this criterion of electrophoretic homogeneity to identify these abnormal proteins, it has been found (1–3) that approximately one-half of a group of one hundred myeloma patients has both a serum and a urine protein abnormality, another one-third of these cases shows only a serum abnormality, with no characteristic proteinuria, and the remaining one-sixth of the cases exhibits a discrete urine protein peak with no abnormal protein peak demonstrable in the serum

The present study was designed to elucidate the inter-relationship between the serum and urine proteins in the first group of cases, *i e*, in those patients with both a serum and a urine abnormality For this purpose, an isotopically-labelled amino acid was administered to a patient with multiple myeloma and the incorporation and turnover of this label was followed in these two proteins (Ms hereinafter designates the abnormal serum globulin, and Mu the urinary [Bence-Jones] protein) If the time-characteristics of the isotope curves obtained from Ms and Mu satisfied the criteria for a precursor-product relationship (Ms as precursor, Mu as product), it would be supportive, although not conclusive evidence for such an inter-relationship

SUBJECT AND METHODS

Case history

The subject (I A.) was a 50-year-old colored female, admitted to the Delafield Hospital in April, 1954, with a ten-month history of increasingly severe low back pain Past medical history was non-contributory In 1951, she was found to have an anemia, the origin of which was obscure. Because of back pain she had been admitted to another hospital in March, 1954, where work-up had revealed Hgb 6.2 Gm, Bence-Jones proteinuria, blood urea nitrogen, 10 7 mg per cent, total serum protein, 10 8 Gm. per cent, A/G, 4.2/6 6, myeloma cells in bone marrow, and x-ray evidence of a pathological fracture of the third lumbar vertebral body

When transferred to the Delafield Hospital one month later, laboratory investigation disclosed Hgb 4 9 Gm., wbc 2,800, neutrophils 55 (15–40), lymphocytes 32, eosinophils 3, monocytes 10, platelets 164 000, non protein nitrogen 23 mg per cent, ESR 160 mm per hr Paper electrophoresis of the serum and urine (Figure 1) confirmed the presence of an electrophoretically homogeneous abnormal protein in both the serum and urine. The serum component (Ms) had the electrophoretic mobility of a γ globulin, Mu was of beta mobility Ms, when stained for carbohydrate by the periodic acid-Schiff technique (4), gave a strongly positive reaction, whereas Mu was Schiff-negative (Figure 1) Iliac bone marrow aspiration revealed 50 per cent myeloma cells Widespread osteoporosis and osteolytic lesions were seen on skeletal survey

Transient symptomatic benefit was obtained from a course of radiotherapy to the lumbar spine and transfusions in May, 1954, but there was roentgenographic evidence of overall disease progression A further palliative course of 1,000 r to the thoracic spine was administered in early September, 1954 No urethane or other chemotherapeutic agent was administered prior to the isotope study Despite two transfusions in the two weeks pre-

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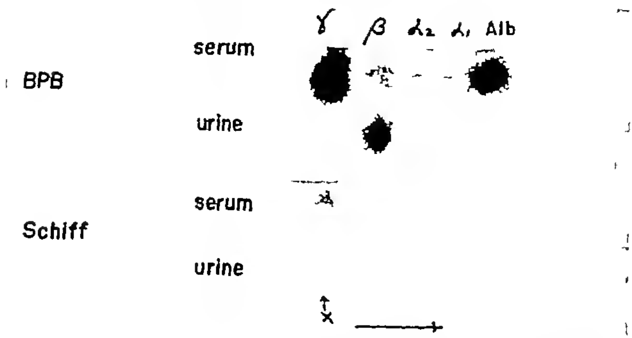

FIG. 1 PAPER ELECTROPHORETIC PATTERNS OF THE SERUM AND URINE OF PATIENT I A., STAINED FOR PROTEIN WITH BROMPHENOL BLUE (BPB) AND FOR CARBOHYDRATE WITH THE SCHIFF TECHNIQUE

"X" indicates the site of sample application, and the arrow designates the direction of migration.

ceding the study the blood count on the day of isotope administration was Hgb., 7 0 Gm. rbc, 2 47 million wbc, 2,500 platelets 88,000 Further data at this time (9/24/54) Non protein nitrogen 43 mg per cent uric acid, 4 1 mg per cent calcium, 12.8 mg per cent.

The isotope study was well tolerated and, throughout the 17-day observation period, appetite and food intake were excellent. An intercurrent cystitis secondary to the presence of an indwelling catheter responded satisfactorily to antibiotics The effect of this cystitis on the qualitative nature of the proteinuria, and the procedural changes necessary for urine protein separations are described below Three transfusions of packed red blood cells from 500 cc. of whole blood were given during the 17-day period.

Following the isotope study a therapeutic trial of cortisone and subsequently a course of urethane were administered with slight subjective (but no objective) evidence of benefit. Urethane was discontinued after 6 weeks (total dose 105 Gm.) because of pancytopenia. Two weeks prior to death, the patient developed nitrogen retention, and despite supportive measures she expired on 28 December 1954

Autopsy confirmed the widespread osseous destruction with myeloma tissue. The kidneys showed the tubular epithelial degenerative changes and the proteinaceous casts considered typical for Bence-Jones protein damage. Fine vacuolization of the tubular epithelial cells was also seen. The immediate cause of death appeared to have been an extensive bilateral lobular pneumonia.

Physico-chemical characteristics of the subject's myeloma serum (Ms) and urinary (Mu) proteins

Figure 2 shows the moving boundary electrophoretic patterns of the whole serum and Figure 3 the electrophoretic and ultracentrifugal diagrams of the isolated serum (Ms) and urine (Mu) proteins Throughout the experimental period the total serum protein concentration remained at 14 Gm per cent with the following percentage distribution of components albumin 15 9 alpha 1 globulin 2.4 alpha 2 globulin 4 9 beta-globulin 6 1 gamma globulin (Ms) 70.7 per cent. Thus, the Ms component was present at a concentration of approximately 10 Gm per cent. The electrophoretic mobility of Ms in Veronal buffer pH 8 6 ionic strength 0 1 was 1.3×10^{-5} $cm.^{2}$ $sec.^{-1}$ $volt^{-1}$ The sedimentation constant $S_{20,w}$ of Ms = 6.0 S Mu had a mobility of 2.7×10^{-5} $cm.^{2}$ $sec.^{-1}$ $volt^{-1}$ and a sedimentation constant $S_{20,w}$ of 3.5 S The mono-dispersity of these proteins is apparent from the

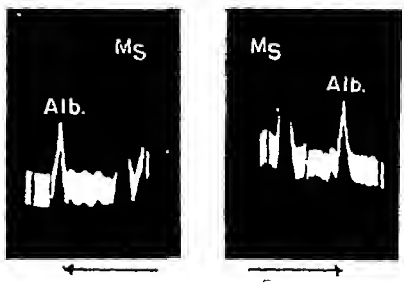

FIG. 2. ELECTROPHORETIC PATTERNS ASCENDING (LEFT) AND DESCENDING (RIGHT) BOUNDARIES OF WHOLE SERUM OF SUBJECT I A OBTAINED IN VERONAL BUFFER pH 8 6

The arrows indicate the direction of migration.

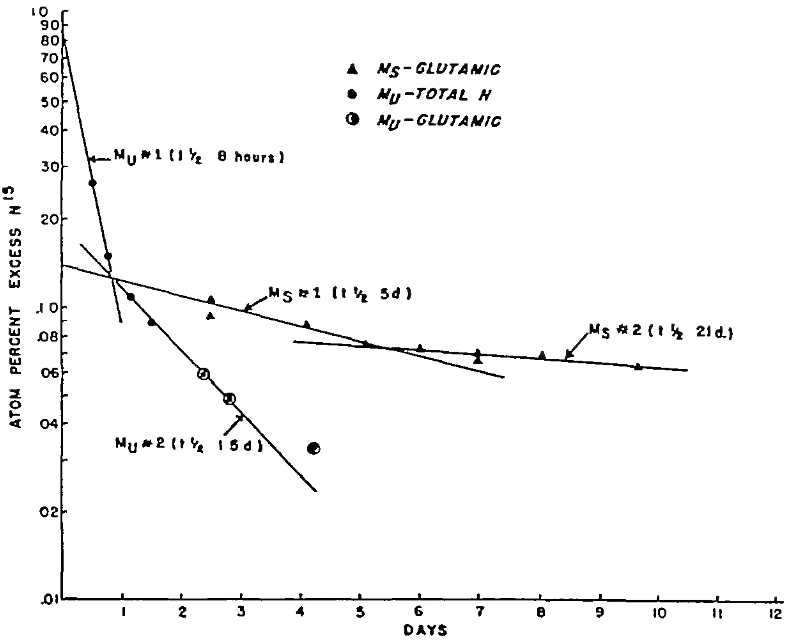

FIG 6 SEMI-LOGARITHMIC PLOT OF N^{15} ABUNDANCE DATA FOR ▲ MS-GLUTAMIC ACID ● MU-TOTAL NITROGEN AND ○ MU-GLUTAMIC ACID

reached until the second day, and the subsequent decline proceeded at a much slower pace than for Mu The isotope curve for Alb (α β) was intermediate in its time-characteristics between these two myeloma protein curves, showing faster rate of decline than Ms, but considerably slower than Mu

Semi-logarithmic plots of the declining portions of the Ms and Mu isotope curves are shown in Figure 6 In both instances, the data appear to show the best fit to two separate exponential functions For Ms, the first exponential (E^1) has a t½ of 5 days, the second (E^2), a t½ of 21 days For Mu, (E^1) has a t½ of 8 hours, and (E^2), of 1 5 days The Alb (α, β) isotope curve (not shown in Figure 6) follows a similar double exponential decline pattern, with the t½ of (E^1) equal to 3 5 days, t½ of (E^2) equal to 12 5 days

The initial rapid rates of decline in isotope concentration in these three proteins (the E^1s) undoubtedly represent the net resultant of several functions of isotope distribution and dilution into amino acid and protein pools of different magnitudes proceeding concomitantly with protein degradation and excretion (Mu) The fact that single exponential functions approximate the data during these early periods is probably fortuitous The second decay functions should more nearly reflect the respective rates of protein degradation, with the probable implicit error of isotope recycling In the case of Mu, there exists the added factor of excretion The extent to which the isotopic label is liberated by protein breakdown back into the metabolic nitrogen pool and reincorporated into these same proteins is impossible to estimate

Figure 7 shows the isotope curves for Ms glutamic, aspartic and total protein nitrogen The time-characteristics of these three curves are essentially similar, supporting the postulate of uniformity of turnover of the formed protein molecule, and, as expected, the isotope abundance in the individual amino acids exceeds the N^{15} concentration in total protein nitrogen It is particularly noteworthy that glutamic N^{15} concentration was uniformly higher than aspartic N^{15}, although aspartic had been administered [3] This concentration

[3] Wu and Rittenberg (5) have noted the same concentration differential, i e, plasma and tissue protein glutamic N^{15} exceeding aspartic N^{15} after N^{15}-aspartic administration This is consistent with the recognized rapid rate at which the amino nitrogen of aspartic acid exchanges with the nitrogen of the metabolic pool Aspartic acid is so rapidly deaminated that its amino group may be considered to behave metabolically like ammonia.

difference for glutamic, aspartic and total protein nitrogen was also found in the Alb (α β) samples (Figure 8)

The Mu samples during the first 48 hours were too small to permit isolation of glutamic and aspartic acids and only total protein N^{15} could be measured When the pooled Mu samples of the third and subsequent days were available for glutamic and aspartic isolations these were performed As indicated in Figures 4 5 and 6 the glutamic N^{15} values appeared to follow the curve of the earlier Mu total N points In these later Mu samples the glutamic N^{15} values and the total Mu protein N^{15} values were not significantly different within the limits of accuracy of the analytic methods

DISCUSSION

A number of physico-chemical studies of myeloma serum and urinary proteins (8–10) have documented a consistent difference in molecular weights of these two constituents Although the serum globulins vary considerably in molecular weight in individual cases they most frequently have been found to have sedimentation and diffusion constants indicative of molecular weights in the range of 160 000 The urinary Bence-Jones proteins by contrast are usually of much smaller size with mean molecular weights in the range of 35 000 to 40 000 The sedimentation constants of Ms and Mu of the patient in this present study are consistent with molecular weights in these respective ranges

A study (4) of the carbohydrate content of myeloma serum and urine proteins in our laboratory indicated that whereas the myeloma serum

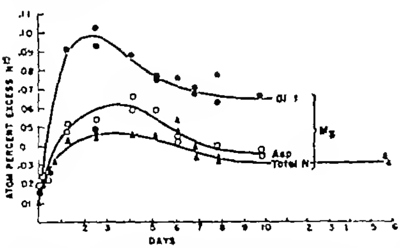

FIG 7 N^{15} ABUNDANCE IN THE ● GLUTAMIC, O ASPARTIC AND TOTAL PROTEIN NITROGEN ▲ FRACTIONS OF Ms

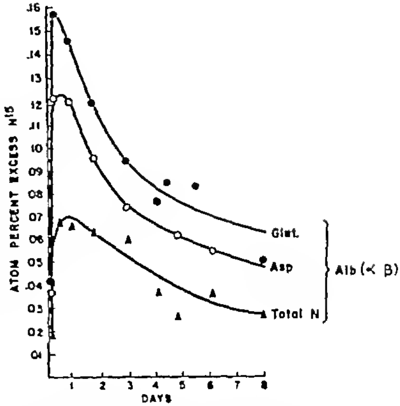

FIG 8 N^{15} ABUNDANCE IN THE ● GLUTAMIC, O ASPARTIC AND TOTAL PROTEIN NITROGEN ▲ FRACTIONS OF ALB (α β)

globulins contained a significant quantity of conjugated Schiff positive material presumably protein bound hexose and hexosamine the urinary myeloma proteins were apparently devoid of these substances These observations were subject to at least three possible interpretations namely (I) that Ms and Mu are two distinct protein moieties independently elaborated and independently metabolized (II) that the urinary protein (Mu) represents a fragment of the serum globulin (Ms) after removal of the conjugated carbohydrate moiety or (III) that the protein excreted in the urine represents a portion of a larger body pool of this material which is functioning as a precursor of the serum globulin i e prior to conjugation with its carbohydrate moiety

If the second postulate were correct i e Ms is the precursor of Mu the isotope concentration maximum in Ms should have preceded and exceeded the isotope maximum in Mu Since just the opposite relationships were observed between these two turnover curves postulate II would appear to be invalid leaving the choice between I and III As will be seen shortly after consideration of other available data this choice cannot, as yet be clearly and conclusively made

Essentially similar results to those herein reported have been obtained by Putnam Hardy

TABLE I

	Labelled compound	Serum globulin		Urinary (Bence-Jones) protein		Albumin
		S_{20}	$T_{1/2}$	S_{20}	$T_{1/2}$	$T_{1/2}$
Putnam and Hardy (11)	NH_2 CH_2 $C^{14}OOH$	6 2 8 8	17–20 d	3 08	18 hours	No data
Hardy and Putnam (12)	$N^{15}H$ CH_2 COOH	6 6 (Cryo-)	? about 20 d	2 14	1 8 d	No data
This paper	HOOC CH CH $N^{15}H$ COOH	6 0	E^1 = 5 d E = 21 d	3 5	E^1 = 8 hrs E^2 = 1 5 d	E^1 = 3 5 d E^2 = 12 5 d

Meyer and Miyake (11–13) in studies using C^{13}-glycine in one patient, N^{15}-glycine in a second, and DL glutamic acid-1C^{14} in a third A comparison of their results, from studies (11) and (12) with the data from the present study, is outlined in Table I Several significant differences in the protocols of these three studies are notable, *viz*, Putnam and Hardy's first subject (11) was receiving urethane throughout the experimental period, the subject of the second study had evidence of advanced renal damage and nitrogen retention, and had also received urethane just prior to the isotope experiment This patient's serum protein was a cryoglobulin with only aspartic acid in the N-terminal position The subject of the study herein reported was selected because of the relatively typical nature of the abnormal serum and urine proteins exhibited, and the apparent freedom from the possible effect of prior therapy or renal damage Despite these differences and the fact that different amino acids were employed, the observed results in these three studies are in essential agreement with regard to the turnover rates of the myeloma proteins It must be recognized that the biosynthetic labelling procedure employed in this study and in the studies of Putnam and his co-workers has, implicit within it, a serious source of error due to reincorporation of the isotope This factor of recycling is primarily operative in the later time periods Accordingly, the calculated half-lives must be considered as only very gross approximations to the true biological life-spans of these protein constituents Within these clearly recognized limitations, however, one may speculate on the possible interpretations of the observed isotope turnover curves of Ms and Mu

The extremely rapid turnover of Mu may in part be explained by the rapid sequestration of this protein in the urine, and, hence, its non-availability for later reincorporation of "recycling isotope" The approximately 15 per cent residual contamination of the later Mu samples with albumin and minimal amounts of alpha globulins due to the intercurrent cystitis might be expected to have introduced an error in the direction of prolonging the observed half-life of the urinary protein, since the serum Alb (α, β) fraction displayed a much slower turnover rate than that found for Mu

An isotope turnover study of the albumin in the urine of a patient with nephrosis, comparing the isotope half-life in urinary albumin with serum albumin of the same subject, would probably represent the best reference with which to evaluate the function of urinary sequestration on the form of the isotope turnover curve Although turnover studies of serum proteins in nephrosis have been reported the urinary proteins have not been examined

The half-life of serum Alb (α, β) as measured in our myeloma subject was approximately 12 5 days This is considerably shorter than the values for albumin of normal subjects reported from other studies (14, 15) which utilized biosynthetic labelling techniques[4] Thus, London (14) obtained a half-life for albumin of 20 days in normal subjects after feeding N^{15}-glycine Masouredis and Beeckmans (15) found comparable albumin half-

[4] These data are more closely comparable to the experimental situation of this study than are the more extensive reports (15, 17, 18) of albumin half-life as measured by *in vitro* labelled I^{131}-albumin The values for half-life of I^{131}-albumin are about one-half to one fifth as long as the values for the biosynthetically labelled protein This shorter life-span of the I^{131}-albumin may reflect a significant alteration (? partial denaturation) of the albumin molecule in the *in vitro* labelling process resulting in a shortened life span

lives of 27 6 days in a patient with polycythemia vera, and 39 4 days in a subject with inactive rheumatic heart disease when they employed orally administered C^{14} glycine Volwiler Goldsworthy MacMartin Wood Mackay and Fremont Smith (16) report a range of 23 to 44 days in half life for albumin in normal subjects when they used orally administered S^{35}-cystine. Whereas the rapid turnover of the Alb (α β) fraction in our myeloma subject may partly be due to the contamination of albumin with alpha and beta globulins of more rapid turnover rates than albumin itself it is suggestive of a true augmentation in the rate of albumin degradation in this derangement of protein metabolism The lack of data on patients with other neoplastic diseases and associated hypoalbuminemia however prevents any speculation regarding the specificity or significance of this phenomenon.

An extensive range of values for the half life of normal gamma globulin has been reported from the several laboratories engaged in these studies As in the case of other plasma protein fraction life span studies differences in labelling techniques and methods of protein fractionation appear to exert a profound influence on final calculated values of turnover rates Thus Volwiler and his co-workers (16) using orally administered S^{35}-cystine obtained gamma globulin half lives of 48 to 85 days in normal subjects and 31 days in a patient with cirrhosis A half life range from 19 to 60 days for this fraction has been reported by Armstrong and his associates (19 20) The half life value of approximately 21 days for the Ms protein herein reported and the comparable values for the myeloma serum globulins reported by Putnam and Hardy (11 12) are not clearly divergent from the available isotope turnover data for normal gamma globulin fractions but again it must be stressed that the differences in labelling and fractionation techniques preclude any reliable comparisons

In conclusion it may be stated that the *relative* rates of turnover of the abnormal serum and urine proteins in a case of multiple myeloma have been studied after the oral administration of N^{15} L aspartic acid in an effort to ascertain whether the urinary protein could be a product or a fragment of the larger molecular sized serum constituent Because the isotope concentration maximum of the urine protein (Mu) preceded and exceeded the isotope maximum in the serum globulin (Ms) the latter (Ms) cannot be construed to have been the precursor of the former (Mu) Having observed similar results in their isotope studies of those abnormal myeloma proteins Putnam Hardy Meyer and Miyake (11–13) have suggested that the opposite inter relationship may exist *i e* that the Bence-Jones urinary proteins may represent precursors or abortive products of serum globulin synthesis This alternative hypothesis is surely worthy of further consideration but the present authors do not believe that the currently available experimental data justify this interpretation It would appear that Mu is rapidly synthesized and rapidly excreted whereas Ms is more slowly elaborated and degraded The experimental evidence fails to establish that either protein is the precursor or product of the other

SUMMARY

1 The rates of incorporation and degradation of serum albumin the abnormal serum (Ms) and urine (Mu) proteins in a patient with multiple myeloma have been studied employing orally administered N^{15} L aspartic acid as the biosynthetic label

2 The turnover rate of Mu was found to be extremely rapid with a half-life of 1 5 days whereas the myeloma serum globulin Ms was found to have a much slower turnover rate (half life = 21 days)

3 The isotope concentration maximum in Mu preceded and exceeded the isotope maximum in Ms

4 These data are interpreted as being incompatible with the hypothesis that the larger molecular sized serum globulins of myeloma are precursors of the smaller urinary (Bence Jones) proteins They also fail to establish the opposite inter relationship *i e* that the Bence Jones proteins are abortive precursors of serum globulin synthesis.

5 Until further data prove to the contrary the thesis that Ms and Mu are separate constituents independently elaborated would seem most readily acceptable

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THE METABOLISM OF AMMONIA AND α-KETO-ACIDS IN LIVER DISEASE AND HEPATIC COMA[1]

By W. H. J. SUMMERSKILL,[2] STANLEY J. WOLFE,[3] AND CHARLES S. DAVIDSON

(From the Thorndike Memorial Laboratory and Second and Fourth (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.)

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A causal relationship between ammonia intoxication and hepatic coma is suggested by the reproduction of the syndrome of impending hepatic coma in sensitive patients with liver disease by substances from which ammonia can be derived (1) and is supported by demonstration of deranged ammonia metabolism in liver disease (2). The frequent finding of elevated peripheral vein ammonia concentrations in hepatic coma (3) has encouraged incrimination of ammonia in the genesis of the syndrome (4, 5). Conversely, reservations have been expressed that such results may signify no more than impaired nitrogen metabolism secondary to liver disease (6, 7). More recently Bessman and Bessman (8) have questioned the validity of observations based on peripheral vein blood alone by demonstrating significant arterio-venous ammonia difference in hepatic coma. Moreover, a close relationship has been reported between arterial concentrations and neurological status in a patient with this complication (9).

The purposes of this paper are: 1) Further assessment of the significance of arterial concentrations and A-V ammonia differences in hepatic coma, directing particular attention to the effect of eliminating from the diet nitrogenous substances from which ammonia may be derived; and 2) Investigation of a possible relationship between disordered ammonia metabolism and the elevated blood pyruvate and α-ketoglutarate concentrations reported in liver disease (10, 11). Arterial concentration and tissue metabolism of ammonia have been compared with the neuropsychiatric state and with the protein intake in uncomplicated liver disease and hepatic coma. Simultaneous estimations of blood pyruvate and α-ketoglutarate were performed. The relationship of the keto-acids to ammonia metabolism was further studied in a smaller group of patients with liver disease by measuring blood concentrations of these substances in response to the administration of ammonium chloride.

MATERIAL AND METHODS

Patients. Twenty-seven patients (16 male and 11 female) in impending hepatic coma or coma were studied. Ages were distributed between 29 and 70 years. Twenty patients had cirrhosis associated with chronic alcoholism. The etiology of cirrhosis was uncertain in three patients. One patient had hemochromatosis and another Wilson's disease. One patient had an hepatic lymphoma and the remaining patient had carbon tetrachloride poisoning. Liver failure, evident from progressive jaundice and severe ascites, was judged the basis of hepatic coma in 16 patients. Other factors precipitating coma included major gastrointestinal hemorrhage (5 patients), acute pyogenic infections (3 patients), intolerance of nitrogenous substances with deterioration of liver function (2 patients with portacaval anastomoses), paracentesis abdominis (1 patient) and a major surgical operation (1 patient).

Eleven patients with cirrhosis in the absence of hepatic coma were also studied (10 chronic alcoholics and 1 in whom the etiology of liver disease was uncertain). A group of 18 control subjects without evidence of hepatic, renal or metabolic disorder was recruited from hospital patients and staff.

The diagnosis of liver disease was made on clinical and biochemical grounds; histological confirmation from biopsy or autopsy specimens was available in 27 patients, including 18 of the 20 patients who died in hepatic coma.

Neuropsychiatric assessment. Patients in coma (including impending hepatic coma) were examined daily or more often, assessment being based on the clinical syndrome reported by Adams and Foley (12) and assisted by EEG records when necessary. The charac-

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[2] Rockefeller Travelling Fellow of the Medical Research Council of Great Britain.

[3] Public Health Service Research Fellow of the National Heart Institute.

TABLE I

Cerebral (arterial-jugular bulb difference) and peripheral (arterial-peripheral vein difference) uptake and release of ammonia during terminal two days of hepatic coma *

	Patient and diagnosis	Serum bilirubin (mg /100 ml)	Ascites (0-++++)	Arterial NH_3N (µg /100 ml blood)	A-V difference Ammonia uptake (+) or release (−) Cerebral (µg /100 ml)	Peripheral (µg /100 ml)
GA	Cirrhosis of the alcoholic	26 0	+++	275	+15	+61
SU	Cirrhosis of the alcoholic, massive gastrointestinal hemorrhage	18 4	+++	60	+12	+16
ST	Cirrhosis of the alcoholic, massive gastrointestinal hemorrhage	2 9	+++	250	+66	+83
BC	Cirrhosis of the alcoholic, lobar pneumonia	6 4	0	271	+4	+1
MC	Cirrhosis of the alcoholic	11 0	+++	171	−75	−97
GE	Wilson's Disease	1 9	+++	229	−90	+42
BE	Cirrhosis of the alcoholic, massive gastrointestinal hemorrhage	23 6	++	354		+4
EA	Cirrhosis of the alcoholic	22 0	+++	262		−1
MG	Cirrhosis of the alcoholic	19 0	++	200		−11
MS	Hepatic lymphoma, gastrointestinal hemorrhage	0.5	0	114		−4
SP	Carbon tetrachloride poisoning	38 0	+	52		−11
MK	Cirrhosis of the alcoholic	33 1	+++	160		+63

* Although in coma, the patients were not moribund

teristic fluctuation and frequent disparity between psychiatric and objective neurological findings permitted an accurate distinction only between impending coma and coma for the purpose of this report, stuporous patients responding only to strong stimuli being placed in the latter category

Protein intake Protein intake, during or immediately prior to hepatic coma arising in hospitalized patients, was assessed from the ward diet or calculated from the restricted protein diet, usually a low-sodium milk product,[4] on which patients were maintained. Progressive or advanced hepatic coma was treated with exclusion of all nitrogenous substances (protein, drugs, etc.) from the diet and with broad spectrum antibiotics by mouth (7) Chlortetracyclene in doses of 2 to 4 gm. daily was administered for the duration of the neurological syndrome. The possibility of gastrointestinal bleeding was checked by inspection of feces, examination of stools for occult blood and, in fatal cases, at autopsy

Biochemical methods Blood ammonia was determined by a modification (13) of Conway's method (14), blood pyruvate and α-ketoglutarate were estimated using the method of Seligson and Shapiro (15) Blood was drawn without stasis into specially cleaned syringes and was introduced into the Conway units or flasks at the bedside. The accuracy of these methods in our hands, calculated from the standard deviation from mean recoveries, was ± 3 per cent for ammonia, using standards and blanks, ± 6 per cent for α-ketoglutarate and ± 9 per cent for pyruvate. The recoveries for keto acids were carried out using human blood. "Uptake" of ammonia or keto-acids by brain or peripheral tissues was assumed when the differences between concentrations in arterial and appropriate venous blood (A-V difference) were positive and, conversely, release is represented by negative differences

Administration of ammonium chloride Alterations in blood ammonia, pyruvate and α-ketoglutarate concentrations following the administration of ammonium chloride were measured in 5 patients with liver disease, whose clinical and biochemical findings are given in Table III, and in 4 control subjects Three other control subjects received ammonium chloride prior to determination of arterial and peripheral vein ammonia concentrations Ammonium chloride was given by mouth (as non-enteric coated capsules) or intravenously (2 per cent solution in water or sodium chloride infused over 45 minutes) in 3 0 or 4 0 gm. doses Individuals with severely impaired liver function, extensive portal-systemic collateral venous systems or previous evidence of neuropsychiatric sensitivity to nitrogenous substances received the smaller amount by mouth in view of their susceptibility to hepatic coma induced by ammonium salts (1) Control subjects were given ammonium chloride intravenously as use of the oral route would have prevented most of the ammonia from reaching the peripheral tissues owing to its removal from portal blood by a healthy liver (2) Blood specimens (arterial, peripheral venous, or both) were taken immediately prior to administration of the salt and at varied 30 minute intervals up to four hours for determination of blood ammonia, pyruvate and α-ketoglutarate concentrations Values are reported at 0, 30 to 60, 90 to 120 and 180 to 240 minutes, representing actual readings or mean levels where more than one estimation was made in the relevant period.

[4] Lonalac®, Mead Johnson and Company, Evansville, Indiana.

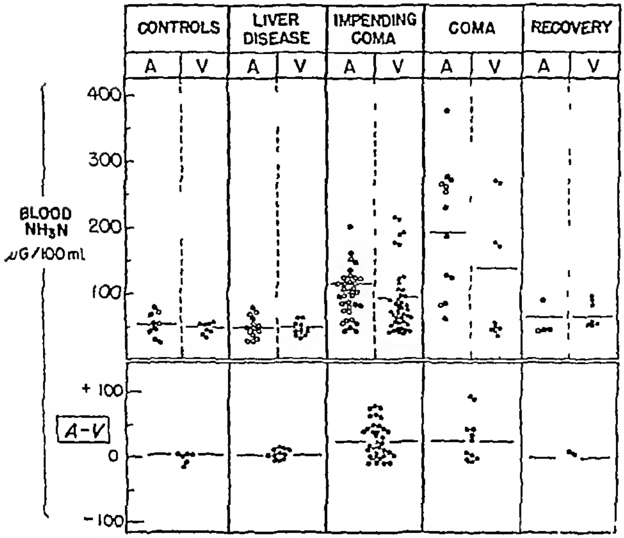


FIG. 1 ARTERIAL (A) AND PERIPHERAL VEIN (V) AMMONIA CONCENTRATIONS WITH A-V DIFFERENCES IN CONTROL SUBJECTS AND PATIENTS WITH LIVER DISEASE WITH AND WITHOUT HEPATIC COMA

RESULTS

Blood ammonia and keto-acid concentrations in hepatic coma

Comparisons were made between the arterial and peripheral vein ammonia concentrations and A-V difference of ammonia in the control group and in patients with liver disease and hepatic coma (Figure 1) Control subjects and patients with liver disease without coma had similar fasting values of ammonia in arterial and venous blood (upper limit of normal 75 µg per 100 ml) and A V differences in both groups indicated that a small and variable uptake or release of ammonia by peripheral tissues occurred in the fasting state During impending hepatic coma the mean arterial concentration (113 µg per 100 ml) was elevated but a quarter of the readings remained within the normal range. The mean concentration was lower in the peripheral vein (92 µg per 100 ml) a half of the values being within normal limits These findings were associated with a greater positive A V difference of ammonia in peripheral tissues in the majority of cases. In patients who had progressed to coma higher mean values of arterial and peripheral vein ammonia were found (193 and 139 µg per 100 ml. respectively) with a smaller proportion of readings (about 10 per cent arterial and 25 per cent venous) still remaining in the normal range The A-V difference was still predominantly positive but relative equilibrium was not uncommon and tissue release of ammonia occasionally of a high degree was sometimes observed Values obtained from patients in the phase of recovery but still exhibiting residual neuropsychiatric disorder showed a return to normal blood ammonia concentrations and tissue equilibrium of ammonia.

Blood α-ketoglutarate and pyruvate values were determined simultaneously with ammonia (Figure 2) Mean concentrations of both substances in fasting patients with liver disease (α-ketoglutarate 20.7 pyruvate 112 µM per liter) were above mean control values (α-ketoglutarate 11 5 pyru

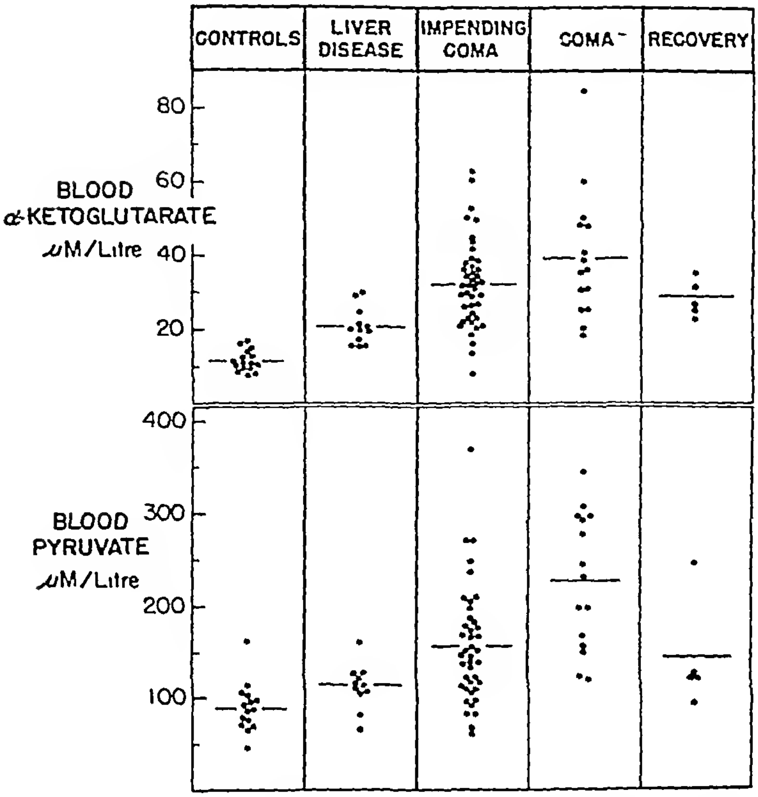
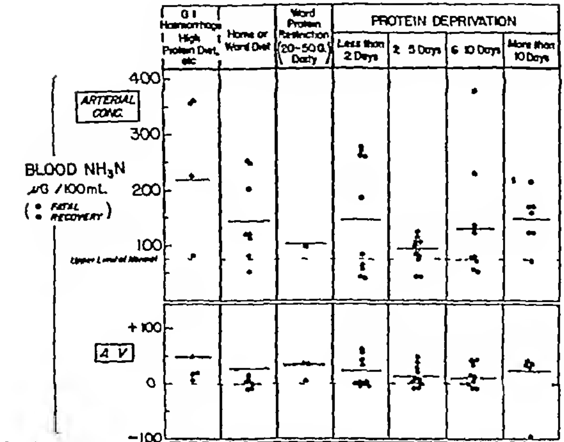


FIG. 2 BLOOD α-KETOGLUTARATE AND PYRUVATE CONCENTRATIONS IN CONTROL SUBJECTS AND PATIENTS WITH LIVER DISEASE WITH AND WITHOUT HEPATIC COMA

ate 89 μM per liter), but there was a considerable overlap in pyruvate values Progressive increase in mean values in impending coma (α-ketoglutarate 32 3, pyruvate 154 μM per liter) and coma (α-ketoglutarate 39 7, pyruvate 225 μM per liter) was observed, but the scatter was wide A third of the α-ketoglutarate and half of the pyruvate values during impending coma and coma were below the upper limits found in patients with uncomplicated liver disease

The influence of nitrogenous material in the intestines on blood ammonia concentration in hepatic coma

Blood ammonia concentrations during hepatic coma were studied in relation to nitrogenous material in the intestine (dietary protein, gastrointestinal bleeding, drugs such as ammonium chloride, etc.) (Figure 3) The highest arterial ammonia concentrations occurred when coma was precipitated by gastrointestinal hemorrhage or by intolerance to nitrogenous substances and the mean levels in patients on conventional home or ward diets exceeded that found when coma was associated with low protein feeding Only one value in each group was within the normal range During treatment by total protein withdrawal and antibiotics, two-thirds of arterial ammonia readings remained high during the first 48 hours, but there was a striking decline towards normal in all patients during the second to fifth-day period, although only three values fell within the normal range In the sixth to tenth-day period, the majority of arterial ammonia concentrations remained at or near normal, but a rise occurred in some patients and a further elevation in the mean value was observed in patients who survived on this regimen for more than 10 days, in the absence of protein feeding, gastrointestinal hemorrhage or uremia Those who recovered and the fatal cases



		G I Haemorrhage High Protein Diet, etc	Home or Ward Diet	Ward Protein Restriction (20-50 G) Daily	Less than 2 Days	2 5 Days	6 10 Days	More than 10 Days
Blood α-Ketoglutarate µM/Litre (Normal 11 8)	Mean ± S D	29.6 ± 13	33 6 ± 20	32.4 ± 66	30 1 ± 9 6	36 ± 12.3	29 4 ± 11	48 3 ± 21
	Total Estimations	6	7	11	10	12	10	8
Blood Pyruvate µM/Litre (Normal 89)	Mean ± S D	156 ± 72	168 ± 41	171 ± 15	192 ± 62	167 ± 72	137 ± 46	190 ± 62
	Total Estimations	6	7	10	10	13	11	8

FIG. 3 ARTERIAL AMMONIA CONCENTRATIONS, A V AMMONIA DIFFERENCES AND BLOOD α-KETOGLUTARATE AND PYRUVATE CONCENTRATIONS DURING HEPATIC COMA IN RELATION TO NITROGENOUS MATERIAL IN THE GASTROINTESTINAL TRACT

had arterial ammonia concentrations which were comparable in the early stages but in patients who survived they returned towards normal more rapidly after protein withdrawal

It was not possible to demonstrate a similar relationship between blood pyruvate or α-ketoglutarate concentrations and intake of nitrogenous material (Figure 3) Mean values for both substances were high at all stages particularly in patients who survived more than 10 days but the scatter was wide.

Uptake of ammonia by peripheral tissue and brain

Peripheral A-V ammonia difference in relation to arterial concentrations greater than 100 µg per 100 ml was compared in control subjects who had received ammonium chloride to patients with liver disease who also had received ammonium chloride or were in the early phase of impending hepatic coma (Figure 4) Although uptake of ammonia occurred in both groups, it was greater in control subjects in relation to arterial concentration and the impaired uptake in patients with liver disease was particularly striking at ammonia concentrations greater than 200 µg per 100 ml No difference was observed between patients with uncomplicated liver disease and those in impending hepatic coma

Uptake of ammonia was the usual finding during the course of hepatic coma (Figures 1 and 3) and was related to arterial concentration Tissue equilibrium or release of ammonia despite high arterial concentrations was not infrequent however and these findings were mainly limited to the terminal phase of coma The arterial concentrations and A-V ammonia differences in 12 unconscious but not moribund patients who were studied during the ultimate 2 days of coma are reported in Table I Cerebral and peripheral A V differences of ammonia were determined

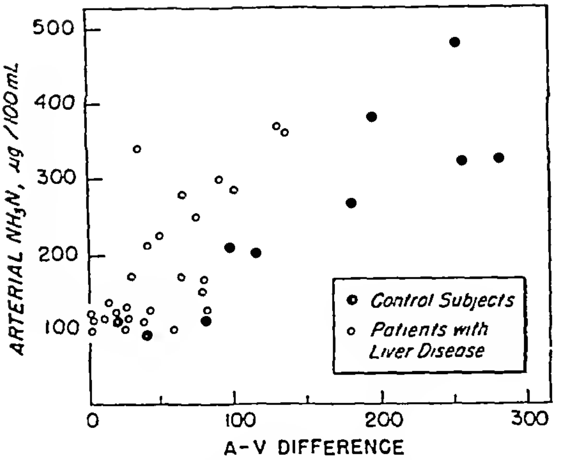


FIG 4 PERIPHERAL TISSUE AMMONIA UPTAKE IN CONTROL SUBJECTS AND PATIENTS WITH LIVER DISEASE

simultaneously in 6 patients Variable uptake of ammonia occurred at both sites in 3 patients (GA, SU, ST), two of whom had massive gastrointestinal bleeding, and a fourth (BC) showed neither uptake nor release at either site despite a high arterial concentration Ammonia release of a high degree was taking place from both brain and peripheral tissues in the fifth patient (MC) and the remaining subject (GE) exhibited release of cerebral ammonia associated with peripheral uptake Of the other 6 patients, in whom peripheral studies alone were performed, only one (MK) had a positive A-V difference, the remainder showing ammonia equilibrium, although arterial concentrations were high in all but one instance

A-V differences of keto-acids

Peripheral A-V differences of pyruvate and α-ketoglutarate were determined on 14 occasions during impending coma, coma or ammonium chloride administration (*vide infra*) Pyruvate levels were higher in the vein in all but one instance, the mean A-V difference being -15 µM per liter (S D $\pm$ 14) There was less evidence of peripheral tissue release of α-ketoglutarate The mean A-V difference was $-2\,1$ µM per liter (S D $\pm$ 2 6), a small positive difference being found on three occasions

Cerebrospinal fluid

Investigation of cerebrospinal fluid (Table II) showed relatively small amounts of ammonia and α-ketoglutarate in control subjects, although pyruvate concentrations were comparable to those in arterial blood In hepatic coma very high ammonia values, comparable with but not clearly related to arterial concentrations, were found With one exception, cerebrospinal fluid pyruvate values reflected and exceeded the arterial concentration in hepatic coma Despite high arterial values, spinal fluid α-ketoglutarate concentrations remained relatively low, but a linear increase with arterial values occurred High pyruvate and α-ketoglutarate values coincided in blood and cerebrospinal fluid but were unrelated to ammonia concentrations at either site

Blood keto-acid concentrations in response to ammonium chloride administration

Control subjects demonstrated no constant alteration in mean blood α-ketoglutarate concentra-

TABLE II

Arterial and cerebrospinal fluid concentrations of ammonia, pyruvate and α ketoglutarate in control subjects and in terminal hepatic coma

	Ammonia (NH_3N µg /100 ml)		Pyruvate (µM/Liter)		α Ketoglutarate (µM/Liter)	
	Arterial	C S F	Arterial	C S F	Arterial	C S F
Controls (7 subjects)						
Mean ± S D	52	20 ± 6*	112	107 ± 25	11 5	1 1 ± 0 8
Hepatic coma						
Patient M C	171	408	295	275	85.5	6 5
R. Y	122	161	190	218	36 5	3 9
B E	71	76	111	206	34 5	2 6
G E	229	92	92	123	27 7	2 0

* From Clarke, Parsons Smith, Sherlock, and Summerskill (29)

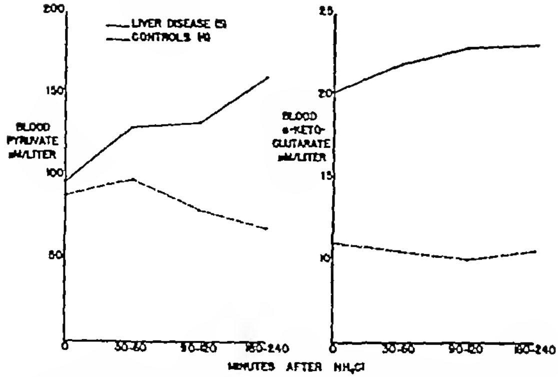


FIG. 5. MEAN BLOOD PYRUVATE AND α-KETOGLUTARATE CONCENTRATIONS FOLLOWING ADMINISTRATION OF AMMONIUM CHLORIDE TO CONTROL SUBJECTS AND PATIENTS WITH LIVER DISEASE

tions following the administration of 4 gm ammonium chloride intravenously but a late fall in mean pyruvate level was evident (Figure 5) Similarly individual α-ketoglutarate values showed little variation from the fasting reading in this group but pyruvate concentrations after an early increase in 2 patients declined as the test continued (Table III) All patients with liver disease exhibited a rise of pyruvate and α-ketoglutarate concentration both in mean values (Figure 5) and individual readings (Table III) The more striking elevations occurred in pyruvate levels which progressed steadily to a maximum at the 3 to 4-hour determination One patient (B A) in whom sensitivity was anticipated owing to chronic nitrogen intolerance following portacaval anastomosis showed a 95 per cent increase in pyruvate concentration after only 3 0 gm. of ammonium chloride by mouth but other patients receiving the drug by the oral route (S.E., T.A) exhibited smaller augmentations of blood pyruvate values than those receiving intravenous ammonium chloride (B U., C O) despite comparable or worse liver function

The mean elevation of blood α-ketoglutarate concentrations in patients with cirrhosis in response to ammonium chloride was less but of a magnitude beyond the error of the method It was a constant finding in every patient (Table III) and the greatest increase of α-ketoglutarate concentration occurred by 30 to 60 minutes thus preceding the greatest alteration in pyruvate values

Ammonia uptake by peripheral tissues following the administration of ammonium chloride was demonstrated by elevation of blood ammonia levels and positive A V ammonia differences in both groups (Table III) coinciding in patients with liver disease with elevation of α-ketoglutarate and preceding the major rise in pyruvate levels The return to fasting values occurred earlier in patients receiving the drug intravenously but further assessment of individual tolerance was not considered relevant to the study

The sequence of events is demonstrated in Figure 6 Following oral ammonium chloride rapid elevation of blood ammonia levels was accompanied by a rise in peripheral tissue uptake of ammonia and α-ketoglutarate concentration The steady rise of blood pyruvate values continued after blood levels of ammonia and α-ketoglutarate had declined and after the patient returned to his initial neuropsychiatric state

DISCUSSION

Increased peripheral vein ammonia concentrations reported in patients with liver disease uncomplicated by hepatic coma (2, 3 5 7 16) are

TABLE III

*Blood pyruvate, α ketoglutarate and ammonia concentrations following * ammonium chloride administration (4 0 gm intravenous infusion unless otherwise indicated) in control subjects and patients with liver disease*

Patient Age Sex	Serum bilirubin (mg %)	Ascites	Impending coma	Blood pyruvate (μM/Liter) 0	30–60	90–120	180–240	Blood α ketoglutarate (μM/Liter) 0	30–60	90–130	180–210		Blood ammonia† (NH_3N μg /100 ml) 0	30	60	90	120	180
Control subjects																		
A N , 29, F				86	62	55	53	10 5	8 2	8 8	9 9							
S I , 16, M				66	134	80	81	8 1	10 7	8 6	9 7	A	27	388	118	44	34	
												A V	−15	+196	+20	−18	−10	
F L , 52, M				114	97	103	66	16 0	13 7	13 7	13 5							
C R , 66, M				92	105	85	79	10 0	9 4	9 4	9 4							
			Mean	89	99	81	72	11 1	10 5	10 1	10 6							
Liver disease																		
B U , 44, M	9 0	Transient	0	126	195	198	241	29 8	32 2	32 2	33 3	A	71		373		71	71
												A-V	−6		+129		−9	−5
T A ,‡ 46, F	1 0	+++	0	81	95	113	135	17 9	19 0	21 4	23 2	A	43	279	302		167	
												A V	−14	+66	+90		+80	
C O , 61, M	1 0	0	0	67	78	71	112	14 4	15 6	17 9	18 1							
B A ,§ 65, M	6 0	0	Recurrent	118	160	180	206	22 8	24 9	26 9	23 3							
S E ,§ 70, M	3 0	0	Transient	92	123	110	110	16 9	19 8	17 5	18 4	A	89	320		360		284
												A V	+2	+34		+136		+98
			Mean	97	130	134	161	20 3	22 2	23 2	23 3							

* Time in minutes
† A—Arterial, A V—Arterial Peripheral Vein Difference
‡ Ammonium chloride, 4 0 gm by mouth
§ Ammonium chloride, 3 0 gm by mouth

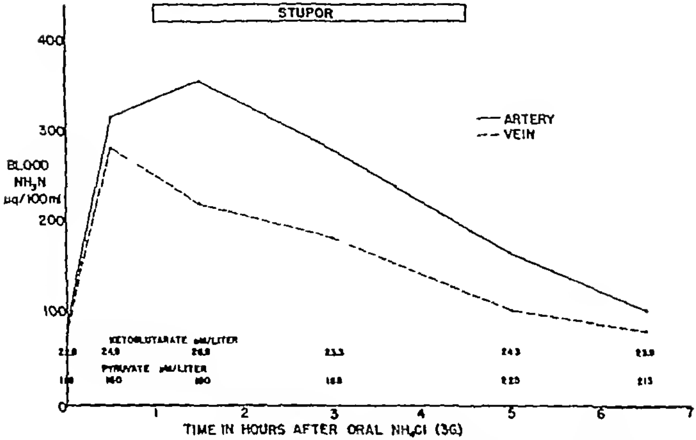


FIG. 6. PERIPHERAL TISSUE UPTAKE OF AMMONIA (A–V DIFFERENCE) AND BLOOD KETO-ACID CONCENTRATIONS DURING EPISODE OF IMPENDING HEPATIC COMA PRECIPITATED BY AMMONIUM CHLORIDE

variably affected by fasting (17 18) but in our patients both arterial and venous values were normal under such circumstances Impaired ammonia tolerance in liver disease (2), however permits abnormal augmentations of blood ammonia following the ingestion of protein (19) blood (20) and other nitrogenous material (1) The highest ammonia concentrations in coma itself were also found by us to be related to these factors During treatment with protein withdrawal and oral broad spectrum antibiotics which presumably reduces the formation of ammonia and other toxic substances by suppression or change of intestinal flora (7 21 22) arterial ammonia concentrations fell towards but seldom to normal. Simultaneously clinical improvement often occurred and the decline in arterial ammonia values was earlier and greater in patients who subsequently recovered

The finding of rising arterial ammonia concentrations later in the course of fatal cases despite continued treatment and the absence of gastrointestinal bleeding or uremia could only sometimes be related to release of ammonia from peripheral tissues or brain Possible additional sources of ammonia include the kidneys which function abnormally in hepatic coma (23), and the failing liver, as high ammonia levels have been reported in renal and hepatic vein blood in hepatic coma (7, 16) The parent compound is unknown and extensive investigation of the phenomenon of ammonia release from mouse brain failed to identify its origin (24, 25) It is relevant however, that Diamox® may liberate ammonia from the brain by direct inhibition of enzyme function with elevation of arterial ammonia levels and the production of impending hepatic coma (18), and it also releases ammonia from the kidney into the renal vein (26)

The high incidence of elevated arterial ammonia concentrations in hepatic coma, with a terminal rise in fatal cases or a prompt fall on recovery supports incrimination of ammonia intoxication in the genesis of the condition However the relationship between clinical status arterial ammonia concentrations and ammonia uptake by brain and muscle reported by others (8 9) is at variance with the frequently poor correlation in our patients The occasional finding of normal values in coma also demands consideration

Ammonia uptake by the brain resulting in depletion of available α-ketoglutarate (27) has been

suggested as the basic disorder of cerebral metabolism in hepatic coma (8), but our findings suggest that removal of ammonia may be accomplished without injury to the organism and that clinical deterioration is more readily related to failure of this removal system Thus, although uptake of ammonia at the periphery occurred in the earlier stages of coma, the efficiency of this process was impaired relative to normal capacity, usually to an extent that could not be explained by increased blood flow in hepatic disease Under these circumstances, progressive deterioration in ammonia removal would result in negligible uptake, equilibrium or even release of ammonia by peripheral tissues and brain, despite high arterial concentration Such findings were, in fact, characteristic of the late stages of coma in our patients

An increased metabolic load is thrown on brain, muscle and other tissues by the abnormal and prolonged rises in blood ammonia which follow absorption of ammonia from the portal vein in some patients with liver disease (2) This may explain the impairment of peripheral ammonia uptake which was found even in the absence of hepatic coma, and stresses the importance of these adjuvant sites of ammonia removal The pre-existing efficiency of these pathways may therefore determine the onset of coma in some instances, and minor overloading could account for the personality changes (28) and EEG abnormalities (29) sometimes observed in liver disease in the absence of objective neurological changes The threshold of coma, depending on the magnitude of the insult and previous efficiency of cerebral and peripheral tissue ammonia removal is therefore unlikely to be closely related to blood ammonia concentrations, which reflect ammonia derived from the gastrointestinal tract modified by endogenous uptake and release at various sites

Evidence of derangement of intermediary metabolism by ammonia was obtained by the administration of ammonium chloride to patients with liver disease The elevation of blood pyruvate and α-ketoglutarate in response to this procedure suggests that the high keto-acid values found in hepatic coma were also related to impaired ammonia metabolism, although imperfect metabolism by a failing liver (30) may be an additional factor Biochemical interpretation of these changes is difficult as pyruvate and α-ketoglutarate may be involved simultaneously in more than one reaction

The rise in pyruvate and α-ketoglutarate concentrations in response to ammonium chloride infusion in liver disease is compatible with a defect in intermediary metabolism (11) It is therefore relevant that Amatuzio, Shrifter, Stutzman, and Nesbitt (31) demonstrated accumulation of blood pyruvate in response to glucose infusion in hepatic coma, a finding which would be in accord with inhibition of final glucose oxidation by the high blood ammonia content in this condition The delayed fall in pyruvate values found in control subjects receiving ammonium chloride could result from utilization of pyruvate in the tissues for transamination reactions

Additional biochemical findings in hepatic coma compatible with deranged ammonia metabolism include high blood concentrations of glutamine (32), asparagine (33), and other amino acids, all of which may reflect increased amidation and transamination involved in disposal of ammonia Precise details of the metabolic disorder still remain uncertain and our investigation failed to support or exclude the theory of cerebral α-ketoglutarate depletion (8) Augmentation rather than removal of peripheral blood α-ketoglutarate occurred in response to ammonium chloride administration in patients with liver disease and greatly increased amounts were available in the blood in hepatic coma Nevertheless, the "blood-brain barrier" (34) may prevent replenishment of this keto-acid in the brain from peripheral blood (8) Examination of the spinal fluid revealed comparatively small quantities of α-ketoglutarate, which is compatible with the hypothesis that it passes from blood to spinal fluid with difficulty, but the higher concentrations present in hepatic coma fail to exclude its availability to the brain

Certain practical points are emphasized by this investigation It was confirmed that similar clinical and biochemical abnormalities occur in hepatic coma regardless of the etiology of the liver disease or nature of the precipitating factor (7) Blood ammonia estimations, particularly arterial concentrations, may be of diagnostic assistance in the fasting patient, but the close relationship postulated between arterial values, tissue uptake of ammonia and clinical status (9) was unreliable be-

cause blood ammonia concentrations reflect the amount of ammonia entering the circulation from the gastrointestinal tract and elsewhere modified by release as well as uptake in various tissues and the relationship between these factors is variable. The results of this investigation suggest the employment of antibiotics in treatment of hepatic coma (35) Glutamic acid also reduces blood ammonia values (36 37) and its variable effect as a therapeutic agent may in part be due to relative impotence in the presence of a large influx of ammonia in the non protein deprived patient or the transient nature of its action in severe liver disease (38) On the other hand, its precursors ammonia and α-ketoglutarate were found in excess in peripheral blood in hepatic coma and elevated concentrations of glutamic acid itself have been reported in this condition (39) It appears from these considerations that assessment of any agent in the therapy of hepatic coma should be compared with the effect of protein deprivation and include estimations of arterial ammonia concentrations while also taking into account the importance of nitrogenous material in the gastrointestinal tract

SUMMARY

1 In 27 patients studied in hepatic coma blood ammonia concentrations were more frequently elevated in the artery than in the peripheral vein but a good correlation with clinical status was evident at neither site. Fasting patients with uncomplicated liver disease had normal blood ammonia concentrations and the height of arterial values in hepatic coma was broadly related to the amount of nitrogenous material in the intestines Values fell towards normal with protein withdrawal and broad spectrum antibiotic therapy but a later elevation occurred in fatal cases despite continuation of this regimen. The origin of this ammonia was uncertain and could only sometimes be attributed to release from brain or muscle.

2 Uptake of ammonia by peripheral tissues was impaired in liver disease relative to normal capacity During the late stages of coma poor uptake, equilibrium or release of ammonia by peripheral tissues or brain occurred despite high arterial concentrations

3 Elevation of blood pyruvate and α-ketoglutarate values paralleled the high blood ammonia concentration in hepatic coma. A rise in blood concentrations of these keto-acids followed the administration of ammonium chloride to patients with liver disease but did not take place in control subjects It is suggested that the high concentrations of keto-acids in hepatic coma represent a defect in intermediary metabolism due to impaired utilization of ammonia and faulty removal from the blood by a diseased liver Concentrations of pyruvate in cerebro-spinal fluid were comparable to those in arterial blood but relatively small amounts of α-ketoglutarate were found there, although values were above normal in hepatic coma

4 The significance of blood ammonia concentrations in hepatic coma must take into account ammonia entering the system from the gastrointestinal tract uptake or release of ammonia at various sites and the possibility of pre-existing defects in ammonia utilizing systems Protein withdrawal with broad spectrum antibiotics effectively reduced blood ammonia values

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THE INTERNAL DISTRIBUTION OF HYDROGEN IONS WITH VARYING DEGREES OF METABOLIC ACIDOSIS[1]

By WILLIAM B SCHWARTZ[2] KARL J ØRNING[3] AND RICHARD PORTER[4]

(*From the Medical Service New England Center Hospital and the Department of Medicine Tufts University School of Medicine Boston Mass*)

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When a mineral acid is administered to animal or man a substantial fraction is buffered in sites other than blood or interstitial fluid Van Slyke and Cullen (1) first observed that total blood volume could account for the buffering of only one-sixth of a mineral acid load and suggested that buffer substances throughout the body including those of the tissues are utilized in the defense against metabolic acidosis Recent studies in several laboratories have provided more detailed quantitative data on the internal distribution of administered hydrogen ions. Swan and Pitts (2) in nephrectomized dogs and Schwartz Jenson and Relman (3) in normal human subjects have demonstrated that only about one-half of a mineral acid load is buffered in the extracellular space and red blood cells The remaining hydrogen ions exchange with sodium and potassium from tissue and bone and are presumably buffered in these areas

The purpose of this investigation was to determine whether the partition of administered hydrogen ions between the intra and extracellular phases is the same regardless of the magnitude of the acid load or whether variation in the severity of the acidosis results in preferential utilization of one or the other of the buffer compartments In the present experiments the distribution of hydrogen was determined in dogs made progressively acidotic by the intravenous administration of hydrochloric acid. The data demonstrate that when equilibrium is allowed to occur the partition of hydrogen ions between extracellular and intracellular buffers is essentially unaffected by the degree of acidosis

MATERIALS AND METHODS

Experiments were performed in 24 healthy mongrel dogs weighing from 10.5 to 21.8 Kg Small doses of morphine were used for sedation during the preparatory manipulations. The femoral artery and vein were cannulated with polyvinyl catheters An indwelling catheter was inserted in the bladder and a Levine tube was introduced into the stomach and left in place for the duration of the experiment. Heparinized arterial blood was collected anerobically for determination of pH, total carbon dioxide content, sodium potassium, chloride, hematocrit, hemoglobin and total protein. Measurements were made of urinary titratable acid, ammonium, phosphorus sodium, potassium, and chloride. The volume of gastric juice was measured periodically and a 5-cc. aliquot was removed for determination of total acid content. The remainder of the gastric content was promptly returned to the stomach. The analytic procedures employed in this study have been described in a previous paper from this laboratory (4) Following a control period of 30 to 60 minutes during which urine and several blood samples were collected, hydrochloric acid in isotonic glucose was administered intravenously by means of a Bowman infusion pump. Employing concentrations of 0.5 to 1.2 N hydrochloric acid, it was ordinarily possible to limit the amount of fluid given to less than 5 per cent of estimated total body water At the completion of the experiments, most of the animals were sacrificed and the volume and total acidity of the stomach contents determined. The small intestine was also regularly examined, but the volume of fluid was small and no measurement of volume or composition was attempted.

Two types of experiments were performed. In the first group of 14 *continuous infusion experiments* hydrochloric acid was administered without interruption until the animal died or the plasma bicarbonate concentration fell to less than 5 mEq per L. In 11 experiments the rate of acid infusion was roughly 80 microequivalents per kilogram per minute and in three 160 microequivalents per kilogram per minute. In the second group of ten *intermittent infusion experiments* hydrochloric acid was

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[2] This work was done during the tenure of an Established Investigatorship of the American Heart Association.

[3] Supported by a grant from the Royal Norwegian Ministry of Foreign Affairs and by a Fulbright Travel Grant.

[4] This work was done during the tenure of a Research Fellowship of the American Heart Association.

TABLE I

Effects of continuous infusion of hydrochloric acid

Dog No. 98 Weight 14.1 Kg., HCl 0.750 N in 5% glucose infused at approximately 80 microequivalents/Kg./min., Hemoglobin 17.2 Gm./100 cc., Hematocrit 41.5%, Plasma protein 5.8 Gm./100 cc.

A. Experimental observations

Total elapsed time	Plasma pH	Plasma pCO_2	Plasma HCO_3	Plasma Cl	Plasma Na	Plasma K	Urine Flow	Urine pH	Urine NH_4	Urine TA	Urine Cl	Urine Na	Urine K	Gastric juice Volume	Gastric juice Total H
min		*mm. Hg*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*cc./min*		*µEq./min*	*µEq./min*	*µEq./min*	*µEq./min*	*µEq./min*	*cc*	*mEq*
−39	7.37	43	24.2	111	146	3.5									
0	7.37	45	25.4	112	146	3.6	0.35	6.70	9	2	29	18	28	5	0
	HCl infusion begun at 0 min. at 1.35 cc./min														
15	7.28	46	20.7												
30	7.26	41	17.7	118	144	3.6									
60	7.14	38	12.4	124	141	3.9	0.35		8	18	12	6	18	13	0
90	7.06	33	9.1	127	141	4.2									
120	6.94	30	6.3	130	140	5.2	0.63	5.34	22	12	65	26	34	23	0.1
150	6.81	22	3.4	134	138	6.5									
165	6.73	20	2.5												
180	6.68	17	2.0	134	133	8.4	0.37		8	59	47	22	24	0	0
201	Dog died. Gastric content at autopsy 35 cc., containing 0.35 mEq. H^+														

B. Derived data

Total elapsed time	ECF volume*	Hydrogen infused	Distribution of administered hydrogen ions: Urine†	Gastric	ECF HCO_3	Plasma protein	RBC HCO_3	Hgb	ICF	Shifts in IC electrolytes: ΔNa	ΔK
min	*L.*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*
30	2.91	30.3	0	0	20	0.4	1.6	2.7	5	−7	0
60	2.99	30.3	0	0	16	0.4	1.1	3.2	9	−3	−1
90	3.12	30.3	1	0	10	0.3	0.6	2.1	16	−19	−1
120	3.25	30.3	1	0	9	0.4	0.6	3.3	16	−15	−4
150	3.35	30.3	0	0	10	0.5	0.6	3.8	15	−9	−5
180	3.56	30.3	0	0	5	0.5	0.3	4.1	20	−11	−8

* Initial ECF volume estimated as 20 per cent BW (2.82 L.)

† Urine was collected less frequently than blood, and urine acid losses were assumed to be constant over the collection interval.

administered in an interrupted manner, each experiment consisting of a series of 45-minute infusions during which acid was given at the rate of approximately 80 microequivalents per kilogram per minute. A two-hour equilibration period y… lowed after each of the infusion periods. Blood … were obtained … eginning and end of e… ion, and ser… 5 and 120 minutes … been disc… all experiment … lative loss … ice was less … or less o… increment … monium acid) … the adm… Several … on expe… exclud… gastric losses c…

- This … it was fo… tration wh… stopped w…

Calculations

Hydrogen ion balance was calculated in a manner previously described (3). Retained acid was taken as the difference between hydrochloric acid administered and the increment in gastric acid secretion and urine acid excretion (ammonium plus titratable acid). The distribution of hydrogen ion in the buffers of the blood was estimated in the fashion described by Singer and Hastings (5). For these calculations blood volume was taken to be 70 cc. per kilogram of body weight, and red cell and plasma volume were considered to be constant, ignoring the sm… or introduced by withdrawal of blood. Initial … lar fluid volume was taken to be 20 per cent o… t and subsequent alterations estimated … om c… e "chloride space." The buffer con… tr… lar bicarbonate was calculated from … in plasma concentration with the … nnan and plasma water correction … llular buffering was taken as the … retained hydrogen ions and that

buffered by the extracellular space.* Internal exchanges of sodium and potassium were estimated in the usual fashion from chloride space" calculations. Shifts of potassium were interpreted on the assumption that there were no significant changes in nitrogen balance in the course of the experiment. Hemolysis was seen frequently at bicarbonate levels of less than 6 mEq per L., but its effect upon calculated potassium shifts was ignored since the potassium content of dog erythrocytes is only from 4 to 12 mEq. per L. (7)

RESULTS

Part I Continuous infusion experiments

Distribution of hydrogen ions among the body buffers Table IA summarizes the data from one of 14 experiments in which acid was infused continuously The upper half of Figure 1 shows the plasma bicarbonate concentration plotted as a function of time in minutes The rate of decrease in bicarbonate concentration was initially rapid but slowed progressively during the experiment Thus in the first half hour a reduction of 8 mEq per L. occurred but in the last half hour of the study an equal quantity of infused acid reduced bicarbonate concentration by only 1 4 mEq per L Twelve of the 14 experiments demonstrated a similar pattern of change in plasma bicarbonate concentration.

Table IB gives the calculated values for distribution of acid based upon measurements made at regular intervals during the course of the acid infusion Acid distribution between intracellular and extracellular fluid for each period of infusion is shown *non cumulatively* in the lower half of Figure 1 and in Table IB and demonstrates an apparent small intracellular buffer contribution early in the study with a much larger intracellular buffer contribution during the latter part of the experiment. Thus extracellular buffers accounted for 83 per cent and intracellular buffers 17 per cent of the first 2 1 mEq per kilogram of acid administered In contrast, during the final period of the experiment the extracellular space buffered only 34 per cent of the last 2 1 mEq per kilogram of acid administered the intracellular phase now having buffered 66 per cent

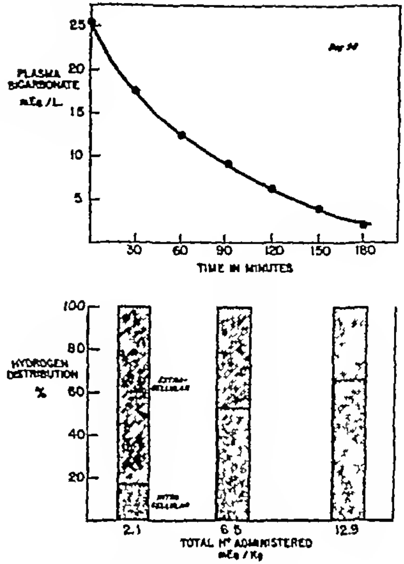

FIG. 1. THE EFFECTS OF CONTINUOUS INFUSION OF HYDROCHLORIC ACID

Plasma bicarbonate concentration is shown in the upper portion. Calculated hydrogen distribution is shown *non-cumulatively* in the lower portion. Note the exponential fall in plasma bicarbonate concentration and the apparent progressive increase in intracellular buffering as acidosis becomes more severe.

In the group of 14 continuous acid infusion experiments the total acid administered varied from 6.8 to 15 8 mEq per Kg a quantity sufficient to reduce plasma bicarbonate concentration in each case to less than 5 mEq per L. Figure 2 summarizes the calculations of intracellular buffering for the entire group of continuous infusion experiments Intracellular buffering for the 14 experiments is plotted against total administered acid but in a *non cumulative* fashion. Each point therefore represents only that buffering calculated to have occurred in the immediately preceding period of infusion The overall pattern for

* Red blood cell buffers make a relatively small and constant contribution to total buffering and have been included with the buffers of the extracellular fluid. The term "intracellular" is used broadly in this discussion to refer to all non-extracellular areas in which hydrogen buffering might occur This probably includes not only muscle and other soft tissues but also bone, which has been demonstrated to have a large labile store of sodium and potassium (6)

TABLE I

Effects of continuous infusion of hydrochloric acid

Dog No 98 Weight 14 1 Kg HCl 0 750 N in 5% glucose infused at approximately 80 microequivalents/Kg /min , Hemoglobin 17 2 Gm /100 cc. Hematocrit 41 5%, Plasma protein 5 8 Gm /100 cc

A Experimental observations

Total elapsed time	Plasma						Urine							Gastric juice	
	pH	pCO_2	HCO_3	Cl	Na	K	Flow	pH	NH_4	TA	Cl	Na	K	Volume	Total H
min		*mm Hg*	*mEq /L*	*mEq /L*	*mEq /L*	*mEq /L*	*cc./min*		*µEq / min*	*µEq / min*	*µEq / min*	*µEq / min*	*µEq / min*	*cc*	*mEq*
−39	7 37	43	24 2	111	146	3 5									
0	7 37	45	25 4	112	146	3 6	0 35	6 70	9	2	29	18	28	5	0
	HCl infusion begun at 0 min at 1 35 cc /min														
15	7 28	46	20 7												
30	7 26	41	17 7	118	144	3 6									
60	7 14	38	12 4	124	141	3 9	0 35		8	18	12	6	18	13	0
90	7 06	33	9 1	127	141	4 2									
120	6 94	30	6 3	130	140	5 2	0 63	5 34	22	12	65	26	34	23	0 1
150	6 81	22	3 4	134	138	6 5									
165	6 73	20	2 5												
180	6 68	17	2 0	134	133	8 4	0 37		8	59	47	22	24	0	0
201	Dog died Gastric content at autopsy 35 cc , containing 0 35 mEq H^+														

B Derived data

Total elapsed time	ECF volume*	Hydrogen infused	Distribution of administered hydrogen ions							Shifts in IC electrolytes	
			Urine†	Gastric	ECF HCO_3	Plasma protein	RBC HCO_3	Hgb	ICF	ΔNa	ΔK
min	*L.*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*
30	2 91	30 3	0	0	20	0 4	1 6	2 7	5	−7	0
60	2 99	30 3	0	0	16	0 4	1 1	3 2	9	−3	−1
90	3 12	30 3	1	0	10	0 3	0 6	2 1	16	−19	−1
120	3 25	30 3	1	0	9	0 4	0 6	3 3	16	−15	−4
150	3 35	30 3	0	0	10	0 5	0 6	3 8	15	−9	−5
180	3 56	30 3	0	0	5	0 5	0 3	4 1	20	−11	−8

* Initial ECF volume estimated as 20 per cent BW (2 82 L)
† Urine was collected less frequently than blood, and urine acid losses were assumed to be constant over the collection interval

administered in an interrupted manner each experiment consisting of a series of 45-minute infusions during which acid was given at the rate of approximately 80 microequivalents per kilogram per minute. A two-hour equilibration period was allowed after each of the infusion periods[5] Blood specimens were obtained at the beginning and end of each acid infusion, and serially 60, 105, and 120 minutes after acid had been discontinued. In all experiments reported the cumulative loss of gastric juice was less than 100 cc. (10 mEq or less of H^+) and urine increment in acid excretion (ammonium plus titratable acid) less than 10 per cent of the administered load. Several additional intermittent infusion experiments were excluded from the study because gastric or urinary losses considerably exceeded these limits

[5] This interval was used because in preliminary studies it was found that the rise in plasma bicarbonate concentration which began immediately after each infusion was stopped, was essentially complete in two hours

Calculations

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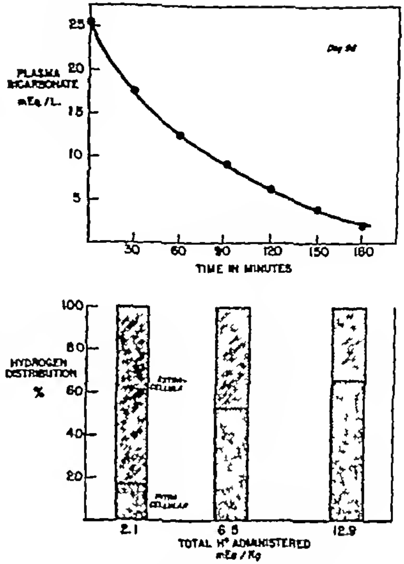

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Total elapsed time	Plasma						Urine							Gastric juice	
	pH	pCO_2	HCO_3	Cl	Na	K	Flow	pH	NH_4	TA	Cl	Na	K	Volume	Total H
min		*mm Hg*	*mEq /L*	*mEq /L*	*mEq /L*	*mEq /L*	*cc/min*		*μEq / min*	*μEq / min*	*μEq / min*	*μEq / min*	*μEq / min*	*cc*	*mEq*
−39	7.37	43	24.2	111	146	3.5									
0	7.37	45	25.4	112	146	3.6	0.35	6.70	9	2	29	18	28	5	0
	HCl infusion begun at 0 min at 1.35 cc /min														
15	7.28	46	20.7												
30	7.26	41	17.7	118	144	3.6									
60	7.14	38	12.4	124	141	3.9	0.35		8	18	12	6	18	13	0
90	7.06	33	9.1	127	141	4.2									
120	6.94	30	6.3	130	140	5.2	0.63	5.34	22	12	65	26	34	23	0.1
150	6.81	22	3.4	134	138	6.5									
165	6.73	20	2.5												
180	6.68	17	2.0	134	133	8.4	0.37		8	59	47	22	24	0	0
201	Dog died Gastric content at autopsy 35 cc, containing 0.35 mEq H^+														

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Total elapsed time	ECF volume*	Hydrogen infused	Distribution of administered hydrogen ions							Shifts in IC electrolytes	
			Urine†	Gastric	ECF HCO_3	Plasma protein	RBC HCO_3	Hgb	ICF	ΔNa	ΔK
min	*L*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*
30	2.91	30.3	0	0	20	0.4	1.6	2.7	5	−7	0
60	2.99	30.3	0	0	16	0.4	1.1	3.2	9	−3	−1
90	3.12	30.3	1	0	10	0.3	0.6	2.1	16	−19	−1
120	3.25	30.3	1	0	9	0.4	0.6	3.3	16	−15	−4
150	3.35	30.3	0	0	10	0.5	0.6	3.8	15	−9	−5
180	3.56	30.3	0	0	5	0.5	0.3	4.1	20	−11	−8

* Initial ECF volume estimated as 20 per cent BW (2.82 L)

† Urine was collected less frequently than blood, and urine acid losses were assumed to be constant over the collection interval

administered in an interrupted manner each experiment consisting of a series of 45-minute infusions during which acid was given at the rate of approximately 80 microequivalents per kilogram per minute. A two hour equilibration period was allowed after each of the infusion periods[5] Blood specimens were obtained at the beginning and end of each acid infusion, and serially 60, 105, and 120 minutes after acid had been discontinued. In all experiments reported the cumulative loss of gastric juice was less than 100 cc. (10 mEq or less of H^+) and urine increment in acid excretion (ammonium plus titratable acid) less than 10 per cent of the administered load. Several additional intermittent infusion experiments were excluded from the study because gastric or urinary losses considerably exceeded these limits

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Calculations

Hydrogen ion balance was calculated in a manner previously described (3) Retained acid was taken as the difference between hydrochloric acid administered and the increment in gastric acid secretion and urine acid excretion (ammonium plus titratable acid) The distribution of hydrogen ion in the buffers of the blood was estimated in the fashion described by Singer and Hastings (5) For these calculations blood volume was taken to be 70 cc. per kilogram of body weight, and red cell and plasma volume were considered to be constant, ignoring the small error introduced by withdrawal of blood. Initial extracellular fluid volume was taken to be 20 per cent of body weight and subsequent alterations estimated from changes in the "chloride space." The buffer contribution of extracellular bicarbonate was calculated from the observed reduction in plasma concentration with the use of a combined Donnan and plasma water correction factor of 1.11 Intracellular buffering was taken as the difference between total retained hydrogen ions and that

TABLE II

Effects of intermittent infusion of hydrochloric acid

Dog No. 88. Weight 21.8 Kg HCl 1 16 N in 5% glucose infused at approximately 80 microequivalents/Kg /min. Hemoglobin 16 4 Gm /100 cc. Hematocrit 52% Plasma protein 6.2 Gm /100 cc.

A Experimental observations

Total elapsed time	Plasma						Urine							Gastric juice	
	pH	pCO_2	HCO_3	Cl	Na	K	Flow	pH	NH_4	TA	Cl	Na	K	Volume	Total H
min		*mm. Hg*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*cc./min*		*µEq./min.*	*µEq./min*	*µEq./min.*	*µEq./min.*	*µEq./min.*	*cc.*	*mEq*
−101	7.39	33	19 1	112	147	3 0									
0	7.36	35	19 0	112	147	3 1	0.21	6.2	11	5	40	45	11	0	0
	HCl infused from 0–45 min at 1 46 cc./min														
45	7.23	26	10.5	122	144	3 6									
105	7.32	26	13 0												
150	7.37	23	13 0												
165	7.35	24	13 1	120	145	4 0	0.50	6 0	19	15	59	36	53	25	1 4
	HCl infused from 165–210 min at 1 49 cc./min.														
210	7 10	17	5.2	132	143	4 6									
270	7.20	19	7.2												
315	7.25	18	7 7												
330	7.26	17	7 4	126	142	6.4	0 16		2	1	8	2	7	5	†
	HCl infused from 330–375 min at 1 44 cc./min														
375	6.87	7	1 2	138	140	7 2									
435	7 07	8	2.2												
480	7 06	9	2 4												
495	7 09	10	3 0	132	139	8.5	0							85	7 0
	Animal sacrificed Gastric content at autopsy 85 cc. containing 7 0 mEq H^+														

B Derived data

Infusion period	ECF volume*	Hydrogen infused	Distribution of administered hydrogen ions							Shifts in IC electrolytes	
			Urine	Gastric	ECF HCO_3	Plasma protein	RBC HCO_3	Hgb	ICF	ΔNa	ΔK
	L.	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*	*mEq*
I	4.62	77	3	1 4	25	0 1	2 4	1.2	44	−29	−12
II	4 95	78	0	0	27	0 4	2.2	3.2	45	−34	−13
III	5 22	76	0	5 6	23	0.8	1 8	6 8	38	−22	−13

* Initial ECF volume estimated as 20 per cent BW (4 36 L.)
† Assumed to have been same as period I

pH and pCO_2. The mean control pH was 7.35 (7.28 to 7 45) pH regularly fell during acid administration in a roughly linear fashion The mean final pH was 6.74 (6 45 to 7 05) pCO_2 regularly fell in a roughly linear fashion during acid administration from a mean initial value of 42 4 mm Hg (32 to 49 mm Hg) to a final mean value of 17 mm Hg (9 to 24 mm. Hg)

Part II Intermittent infusion experiments

Distribution of hydrogen ions among the body buffers Table IIA and Figure 3 summarize data from one of ten experiments in which a two-hour equilibration period was permitted after each of a series of 45-minute acid infusions In the upper half of Figure 3 the periods of acid infusion are represented by the shaded areas Plasma bicarbonate concentration fell sharply during each period of acid administration, rose sharply during the first hour of equilibration and gradually levelled off by the end of two hours In this experiment bicarbonate concentration rose during equilibration by an amount equal to approximately 30 per cent of the initial fall The bicarbonate decrements resulting from the first two acid infusions were of the same order of magnitude but in the last period it was slightly less In each experiment the pattern of change in plasma bicarbonate concentration was essentially the same

the group appeared to be one of increasing intracellular buffer contribution during the first half of the experiment with a gradual levelling off as the intracellular contribution approached 40 to 60 per cent of the load The mean intracellular buffer contribution was 16 per cent of the first increment of acid as compared to a mean intracellular uptake of 55 per cent of the last equal increment In one entirely atypical experiment there appeared to be a steady decrement in intracellular buffering

Extracellular fluid volume Extracellular fluid volume, as estimated from changes in "chloride space," increased steadily during infusion in all experiments, as illustrated in Table I For the entire group the mean total increase in extracellular volume was 15 per cent (6 to 26 per cent)

Plasma electrolyte concentrations Plasma potassium concentration rose progressively in all animals from the mean control value of 3.2 mEq per L (2 5 to 3 6 mEq per L) to a final mean value of 6 6 mEq per L (5 0 to 10 9 mEq per L) The mean increase in concentration was 3 4 mEq per L (0 6 to 7 9 mEq per L)

Plasma sodium concentration decreased slightly but significantly during infusion from the mean control value of 144 mEq per L (139 to 151 mEq per L) to a final mean value of 138 mEq per L (133 to 145 mEq per L)

Exchanges of electrolytes The estimated total shift of potassium from intracellular fluid for the group of experiments had a mean value of 1 33 (± 0 91) mEq per Kg The estimated total shift of sodium from intracellular fluid for the group as a whole had a mean value of 3.26 (± 1 67) mEq per Kg and comprised roughly two-thirds of the estimated intracellular cation loss

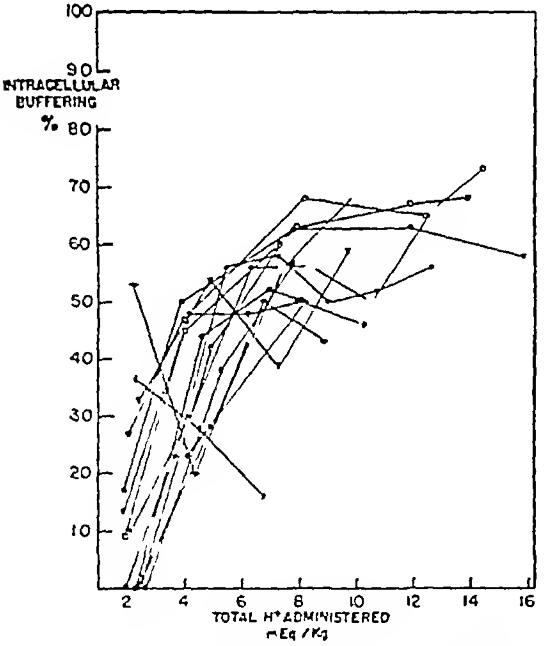

FIG. 2. APPARENT INTRACELLULAR BUFFER CONTRIBUTION DURING CONTINUOUS INFUSION OF HYDROCHLORIC ACID

Intracellular buffering for the 14 experiments is plotted against total administered acid but in a *non-cumulative* fashion. Each point, therefore represents only that buffering calculated to have occurred in the immediately preceding period of infusion. Note the apparent rise in intracellular buffering as acidosis becomes more severe.

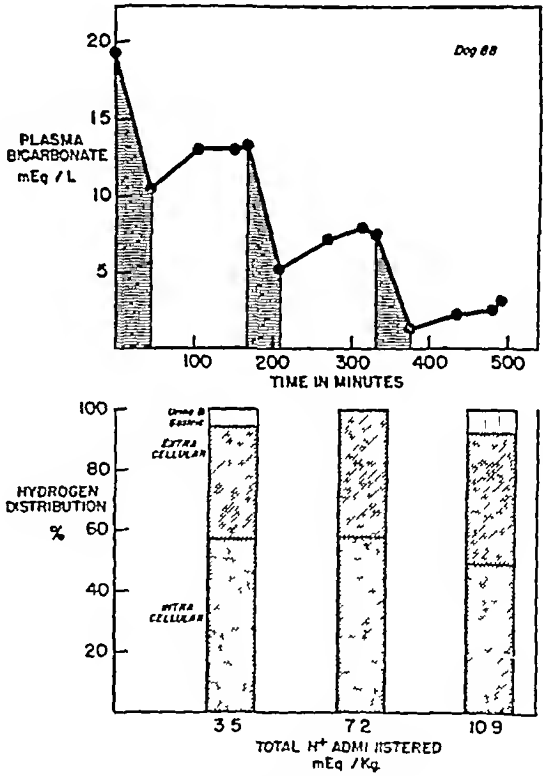

FIG 3 THE EFFECTS OF INTERMITTENT INFUSION OF HYDROCHLORIC ACID

Plasma bicarbonate concentration is shown in the upper portion where the periods of infusion are indicated by the shaded areas Calculated hydrogen ion distribution is shown *non cumulatively* in the lower portion. Note the rebound in plasma bicarbonate concentration during the equilibration period following each infusion, and the roughly constant contributions of extracellular and intracellular buffers at all degrees of acidosis

tracellular volume was 17 per cent (10 to 28 per cent)

Plasma electrolyte concentrations Plasma potassium concentration rose progressively in all animals from the mean control value of 2 9 mEq per L (2.4 to 3 1 mEq per L) to a final mean value of 6.7 mEq per L (5 5 to 8 5 mEq per L) The mean increase in concentration was 3 8 mEq per L (2.9 to 5 4 mEq per L.)

Plasma sodium concentration decreased slightly but significantly during infusion from the mean control values of 144 mEq per L (139 to 150 mEq per L) to a final mean value of 137 mEq per L (131 to 142 mEq per L.)

Exchanges of electrolytes The estimated shift of potassium from the intracellular space had a mean value of 2 58 ($\pm$ 1 16) mEq per Kg Except for a consistently smaller shift with the first infusion, intracellular potassium loss was roughly constant for additional equal increments of acid This is well illustrated by data shown in Table II

Renal excretion of potassium increased in all but the three experiments in which oliguria developed In the seven experiments where urine volume remained at the control level or increased the total potassium loss estimated from the increment above control excretion averaged 2 3 mEq per Kg This urinary loss represented 68 per cent of the potassium calculated to have been displaced from cells

The mean intracellular sodium loss for the group as a whole was 3 81 ($\pm$ 1.61) mEq per Kg and comprised roughly 60 per cent of the estimated intracellular cation loss

For the group as a whole the intracellular cation loss was of the same order of magnitude in each successive infusion period For each experiment the average slope was calculated for a line defining intracellular cation loss as a function of total administered acid. The mean of the average slopes was 1 34 ($\pm$ 2.70) a value not significantly different from zero ($t = 1\ 40$ $p > 0\ 19$) The mean value for hydrogen calculated to have entered cells in this group of experiments was 6 33 ($\pm$ 1.86) mEq per Kg and the mean intracellular cation loss was 6.39 ($\pm$ 2 56) mEq per Kg There is no significant difference between these values ($t = 0\ 05$ $p > 0.90$)

pH and pCO_2. The mean control pH was 7.33 (7.26 to 7 38) For the group as a whole there was a roughly linear fall in pH with successive infusion periods to a final mean value of 7 03 (6.81 to 7 15) At equilibrium after six of 31 infusion periods, pH returned to within 0 02 units of the pre infusion value.

The mean control pCO_2 was 39 mm Hg (34 to 45 mm Hg) For the group as a whole there was a roughly linear fall in pCO_2 with successive infusion periods to a final mean value of 20 mm Hg (9 to 31 mm Hg) In seven of 31 infusion periods however there was no significant change in pCO_2 ($\pm$ 3 mm. Hg)

DISCUSSION

The present studies indicate that the distribution of hydrogen ions among the body buffers is not affected by the magnitude of the acid load The apparent predominance of extracellular buffering early in the course of continuous acid infusion does not represent the 'steady state' response to metabolic acidosis The fact that bicarbonate concentration rises sharply when the acid infusion is stopped reveals that the curvilinear fall in bicarbonate concentration during continuous acid infusion is the resultant of two opposing processes On the one hand the infusion of acid converts bicarbonate to carbonic acid thus tending to lower plasma bicarbonate concentration On the other hand the diffusion of bicarbonate from the interstitial fluid to plasma and the transfer of hydrogen ions from interstitial fluid to the intracellular space both tend to elevate plasma bicarbonate concentration In the continuous infusion experiments this process did not have a chance to approach equilibrium until the late stages of the experiments when the bulk of administered acid had been given adequate time for distribution In the intermittent infusion experiments where equilibrium was allowed to occur at successive points during the study the partition of hydrogen ions between extracellular and intracellular buffers was found to be essentially unaffected by the degree of acidosis

Within individual experiments of the intermittent group there were often rather wide variations from period to period in the estimated contributions of the two buffer compartments the standard deviation from the mean in Figure 4 being $\pm$ 11.6 per cent. It seems probable that the most

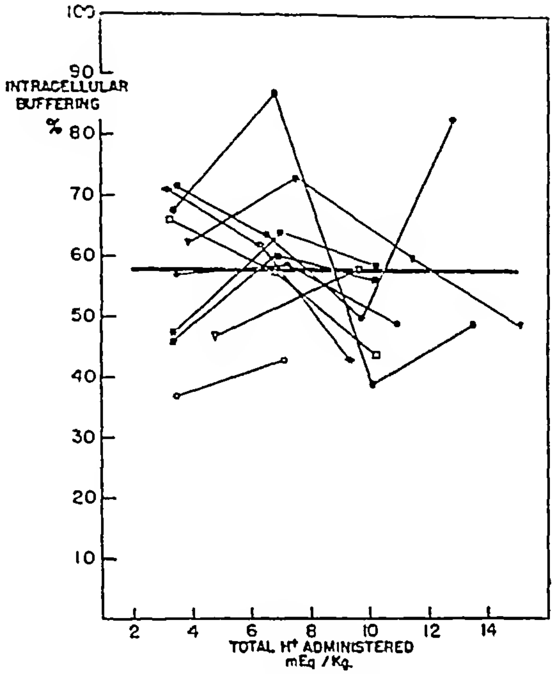

FIG 4 INTRACELLULAR BUFFERING OF SUCCESSIVE ACID INCREMENTS DURING INTERMITTENT HYDROCHLORIC ACID INFUSION EXPERIMENTS

Intracellular buffering for the ten experiments is plotted against total administered acid but in a *non cumulative* fashion. Each point, therefore, represents only that buffering calculated to have occurred at equilibrium following the immediately preceding period of infusion. Note the absence of any consistent trend in intracellular buffering as acidosis becomes more severe, and the random variations about the mean value. Mean of average slopes $= -0.507$ (± 2.37), not significantly different from zero ($t = 0.642$, $p > 0.40$) Mean intracellular buffering $= 57.6$ per cent (standard deviation ± 11.6 standard error ± 2.08)

showing a sharp fall during acid infusion and a rapid rise during the first hour of equilibration During the second hour there was usually only a small additional rise For the group as a whole, taking the mean of all the periods, the reduction in bicarbonate concentration during each infusion was 8 0 mEq per L During equilibration the rise in bicarbonate concentration in most instances tended to be slightly smaller in the second and third periods than in the first period of acid administration The mean values for the net decrement in bicarbonate concentration after equilibration for successive periods were 6 3, 4 6, and 4 6 mEq per L respectively

Table IIB gives the calculated values for distribution of acid based upon measurements made two hours after each infusion Acid distribution between intracellular and extracellular fluid for each infusion period is shown *non-cumulatively* in the lower half of Figure 3 Intracellular buffering varied between 50 and 58 per cent, and extracellular buffering between 37 and 43 per cent during the course of the experiment The remaining fraction of administered hydrogen ions appeared in urine and gastric juice

In the entire group of intermittent infusion experiments, the total acid administered varied from 7 1 to 15 1 mEq per Kg Some animals tolerated only two periods of infusion before developing lethal acidosis, others three and four As will be seen, the amount of acid administered did not affect its pattern of distribution Figure 4 summarizes the calculations of intracellular buffering for the entire group of intermittent experiments Intracellular buffering for the ten experiments is plotted against total acid administered but in a *non-cumulative* fashion Each point, therefore, represents only that buffering calculated to have occurred in the immediately preceding period of infusion The slope for the best-fitting straight line was calculated for each of the ten experiments The mean of the slopes of these lines was -0.507 (± 2.37), a value not significantly different from zero ($t = 0.642$, $p > 0.40$) The mean percentage buffering by intracellular fluid was 57 6 per cent (standard deviation ± 11.6, standard error ± 2.08)

The utilization of extracellular bicarbonate in the group as a whole did not appear to differ significantly with successive periods of infusion For each experiment the average slope was calculated for a line defining extracellular bicarbonate utilization as a function of total administered acid The mean of the ten average slopes was -0.411 (± 1.15), a value not significantly different from zero ($t = 1.07$, $p > 0.30$) The mean buffer contribution of red cells and plasma protein for the 31 infusion periods was 7.1 ± 3.7 per cent of the administered load

Extracellular fluid volume Extracellular fluid volume, as estimated from changes in "chloride space," increased steadily from period to period in all experiments, as illustrated in Table II For the entire group the mean total increase in ex-

of variations in the size of buffer stores depending for example on the relative amounts of fat and lean tissue in a given animal.

In the present study hyperkalemia was observed in every experiment despite, in most instances a normal urine flow and a marked increase in potassium excretion. Previous workers have noted sizable potassium shifts to extracellular fluid when metabolic acidosis was induced in nephrectomized animals (2 8) The present data suggest that in acute metabolic acidosis even with intact kidneys the rate at which potassium is displaced by hydrogen exceeds the kidney s excretory capacity for this ion The degree of hyperkalemia was directly related to the severity of the acidosis although the rise in plasma potassium concentration was generally smallest in the animals with the largest increments in potassium excretion Potassium concentration rose by an average of 3 to 4 mEq per liter but it is probable that elevations of this degree would not be maintained in chronic acidosis where more complete renal compensation could occur It should be noted however that serum potassium concentration tends to be maintained at slightly elevated levels relative to total body potassium stores as long as pH remains low (9)

The mechanism governing the distribution of hydrogen ions through total body buffer stores is not clear from the present experiments It seems likely that hydrogen ion distributes itself across cell membranes according to its electrochemical gradient (10) Thus any change in intracellular-extracellular gradient of hydrogen would tend to produce a shift of this ion The present data show a progressive reduction in extracellular pH as more acid was administered and it seems probable that this increase in hydrogen ion concentration was the major factor in the transfer of hydrogen ions to the intracellular space. There were however exceptions to this pattern in individual experiments where despite a fall in plasma bicarbonate concentration and a sizable movement of hydrogen into cells there was little or no change in extracellular pH Nevertheless hydrogen transfer may still be explained in terms of a change in hydrogen gradient since in these instances extracellular pH was preserved by virtue of a sharp reduction in pCO_2. Since a fall in pCO_2 would probably be accompanied by a rise in intracellular pH even in the presence of a constant extracellular pH the gradient would be altered in a way favoring the inward movement of hydrogen ions Thus, although there is no entirely predictable pattern in the change of either pH or pCO_2, their individual effects on hydrogen gradient may act together to produce roughly the same hydrogen distribution Further studies with control of pCO_2 may serve to clarify this problem

SUMMARY

Hydrochloric acid was administered intravenously to dogs in order to define the internal distribution of administered hydrogen ions with varying degrees of metabolic acidosis. Experiments in which acid was infused continuously appeared to indicate preferential utilization of extracellular buffers in the initial phase with the contribution of intracellular buffers becoming more important as the acidosis increased in severity However, when the acid load was administered intermittently allowing time for equilibrium to occur the partition of hydrogen ions between extracellular and intracellular buffers was found to be essentially unaffected by the degree of acidosis The data indicate that at equilibrium the percentage reduction in plasma bicarbonate concentration provided an approximate index of the percentage reduction in total body buffer stores

ACKNOWLEDGMENT

The authors wish to express their appreciation to Dr Jane Worcester of the Harvard School of Public Health for advice and assistance in the statistical analysis of the data.

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4 Relman, A. S., and Schwartz, W B., The effect of DOCA on electrolyte balance in normal man and

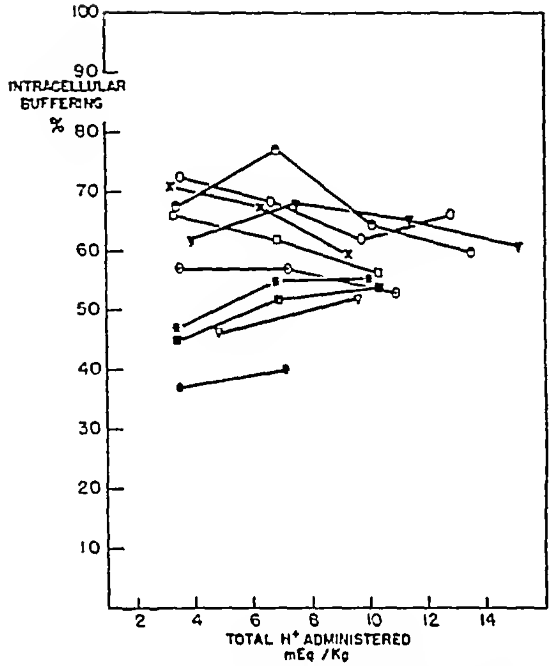

FIG 5 INTRACELLULAR BUFFERING OF TOTAL ADMINISTERED ACID DURING INTERMITTENT HYDROCHLORIC ACID INFUSION EXPERIMENTS

Each point represents the *total* intracellular buffer contribution up to the time of any given equilibrium observation Note that the variation in buffering of the total load by the intracellular area is less than 10 per cent in the course of nine of the ten experiments

important factor in the production of these variations was the secretion and absorption of gastrointestinal juices The single most critical measurement upon which the calculation of hydrogen distribution depends is plasma bicarbonate concentration Sudden transfer of acid between plasma and the gastrointestinal tract by either secretion or reabsorption of gastric juice would markedly influence plasma bicarbonate concentration until equilibrium between plasma, interstitial fluid and tissues had been achieved If such exchanges occurred shortly before collection of the "equilibrium" blood, significant distortion of the calculations of buffer distribution would result Although total gastric losses were modest in the present series of experiments, changes in gastric acid content from period to period were probably large enough to have introduced significant artifacts and were probably the chief cause of the random variations in calculated buffer distribution shown in Figure 4 On the other hand, urinary acid losses were constant enough presumably to have been of lesser significance

Errors in calculation of extracellular fluid volume are probably of minor consequence in the interpretation of these experiments As estimated from "chloride space," extracellular volume increased steadily in the course of every experiment The mean increase in extracellular fluid agreed closely with the change in inulin and radiosulfate space found by Swan and Pitts following a single acid infusion in nephrectomized dogs (2) Although an assumed figure of 20 per cent of body weight was used for initial extracellular volume, substitution of other values between 15 per cent and 30 per cent does not significantly alter the calculated pattern of acid distribution

The sources of error considered above are probably of the same absolute magnitude regardless of the quantity of acid administered For this reason their relative importance in affecting the estimate of total hydrogen distribution based on a single measurement of plasma composition will be progressively less as acidosis increases Thus when we consider the distribution of the *total* acid load administered up to any given time of observation (Figure 5) rather than the distribution of only the small increments of acid added within each period, the variation within each experiment is much smaller than that shown in Figure 4 In nine of the ten experiments the variation in intracellular buffering was less than 10 per cent In metabolic acidosis the plasma bicarbonate concentration is affected by the total load of acid buffered in the extracellular space up to the time of blood sampling Figure 5 suggests that at equilibrium in any given subject the quantity of acid buffered extracellularly bears a reasonably close relationship to the acid buffered within the cells It follows, therefore, that a single bicarbonate determination will give a satisfactory though approximate estimate of the per cent reduction in total body buffer reserves The rather large differences in distribution of acid between experiments (Figure 5) does not affect this conclusion, but it does suggest the difficulty of estimating the quantity of acid responsible for producing a given reduction in plasma bicarbonate concentration The differences in the relative distribution of acid from subject to subject may in part have been the result

of variations in the size of buffer stores depending for example on the relative amounts of fat and lean tissue in a given animal.

In the present study hyperkalemia was observed in every experiment despite in most instances, a normal urine flow and a marked increase in potassium excretion. Previous workers have noted sizable potassium shifts to extracellular fluid when metabolic acidosis was induced in nephrectomized animals (2 8) The present data suggest that in acute metabolic acidosis even with intact kidneys the rate at which potassium is displaced by hydrogen exceeds the kidney's excretory capacity for this ion The degree of hyperkalemia was directly related to the severity of the acidosis although the rise in plasma potassium concentration was generally smallest in the animals with the largest increments in potassium excretion Potassium concentration rose by an average of 3 to 4 mEq per liter but it is probable that elevations of this degree would not be maintained in chronic acidosis where more complete renal compensation could occur It should be noted however that serum potassium concentration tends to be maintained at slightly elevated levels relative to total body potassium stores as long as pH remains low (9)

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ANNOUNCEMENT OF MEETING

The 49th Annual Meeting of the American Society for Clinical Investigation will be held in Atlantic City, New Jersey, on Monday, May 6, 1957, with headquarters at the Chalfonte-Haddon Hall The scientific session will begin at 9 A M at the Steel Pier Theater

A STUDY OF THE MECHANISM OF SECRETION OF THE SODIUM-RETAINING HORMONE (ALDOSTERONE) [1, 2]

By JOHN H. LARAGH and HERBERT C. STOERK

(From the Department of Medicine College of Physicians and Surgeons Columbia University the Presbyterian Hospital in the City of New York and the Merck Institute for Therapeutic Research Rahway N J)

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Aldosterone which has been isolated from the adrenal cortex and chemically characterized is the most powerful sodium retaining hormone among the known naturally occurring adrenal steroids (1) As such, it seems probable that it plays a dominant part in the regulation of sodium and potassium metabolism

There is considerable evidence that aldosterone may be unique among adrenal steroids in that its rate of secretion is largely independent of anterior pituitary control. This is suggested by the absence of atrophy of the zona glomerulosa in rats and dogs after hypophysectomy (2 3) by the persistence of aldosterone in the adrenal venous blood of hypophysectomized dogs (4) and by the observation that some patients with acute pituitary insufficiency induced by total hypophysectomy conserve sodium normally (5) and excrete normal amounts of salt retaining hormone in the urine (6)

In 1950 Luetscher Neher Wettstein, and Curtis (7 8) first reported increased sodium retaining activity in the urine of patients forming edema In subsequent studies this group has identified the active substance in the urine as aldosterone. In addition Luetscher and Axelrod (9) have reported increases in the excretion of hormone in normal subjects in response to short periods of sodium deprivation The increase occurs without apparent alteration in the serum sodium concentration.

The present study was designed to examine the effects of changes in the dietary intake and of associated changes in the serum concentration of sodium and potassium ions upon the urinary excretion of the salt retaining hormone Certain aspects of the relationship between the distribution of sodium and potassium ions have been utilized to produce alterations of serum K and Na. Earlier studies have demonstrated that the administration of potassium to sodium-depleted dogs and human subjects produces a marked and sustained hyperkalemia which is not observed in subjects in normal electrolyte balance (10 11)

The present report demonstrates that potassium ingestion, when accompanied by a rise in the serum potassium may be associated with a pronounced increase of sodium retaining activity in the urine. A fall in the serum sodium concentration *per se* does not appear to produce a comparable effect

METHODS

The experiments were carried out on trained, unanesthetized mongrel dogs housed in metabolism cages. The animals were fed a synthetic diet of fixed electrolyte content containing casein, lard, dextrin, dextrose, vitamin and mineral supplements and agar agar to provide approximately 80 calories per kilogram per day The basal diet contained less than 1.8 mEq of Na and less than 0 44 mEq of K per day When KCl was given it was administered either as a 20 per cent solution by stomach tube or incorporated into the diet. Urine was collected daily and pooled in 48-hour lots. After removal of 10-ml. aliquots for estimation of Na K and Cl the urines were stored at − 76 C. Stool specimens were not saved for analysis but none of the dogs had diarrhea at any time. Methods for the estimation of serum and urinary sodium and potassium and chloride have been described previously (10)

For estimation of sodium retaining activity the thawed urine specimens were adjusted to pH 1 with 6 N HCl and extracted continuously for 24 hours with 350 ml. of redistilled methylene chloride in a Wolfe Hirschberg extractor The methylene chloride extract was washed successively five times with 50-ml. portions of 0 1 N NaOH and five times with 50 ml. of distilled water and taken almost to dryness at reduced pressure in an atmosphere of nitrogen. The extracts were dried further over P_2O in a vacuum desiccator at room temperature and then stored at − 4 C. At the time of assay the dried

[1] This paper was presented at the 47th Annual Meeting of the American Society for Clinical Investigation, May 2, 1955

[2] This work was supported in part by a grant from the U S Public Health Service, National Heart Institute (USPHS H 1275)

extract was first dissolved in 4 ml of 50 per cent ethanol and dilutions of 1 16 and 1 80 were made.

The bioassay was carried out on male rats, adrenalectomized 24 hours before the test. After adrenalectomy the rats were offered distilled water ad libitum and a low sodium diet (Na 0 001 per cent, K 0.3 per cent) At the start of the assay 5 ml of normal saline were injected intraperitoneally, and the bladder was emptied by electric shock. The animals were injected subcutaneously with either desoxycorticosterone acetate (DOCA) standards or unknowns in 0.25 ml of 30 per cent ethanol, and placed in groups of three rats in metabolism cages Duplicate groups of three rats were used for each DOCA standard (2, 10 and 50 micrograms) and for each unknown sample in 1 16 and 1 80 dilution Human urine samples were handled similarly except that 24-hour collections were used, and extracts were assayed at 1 16, 1 80 and 1 400 dilutions When samples were strongly active at all dilutions throughout the range tested, appropriate additional five fold dilutions were employed until a suitable end-point could be obtained. Further dilutions of inactive samples were tested since, with excessive amounts of aldosterone (near or greater than 10 micrograms per rat), sodium retention may not occur with this procedure.

At the end of a five-hour collection period the rats were forced to void by electric shock, the total urine volume was measured (4 to 10 ml per group), and the amount of sodium excreted determined by flame photometry The amounts of sodium excreted by the groups injected with DOCA were plotted on an arithmetic scale against the logarithm of the dose of DOCA (2, 10, 50 μg) The standard curve thus obtained serves for estimation of the sodium-retaining equivalents of the unknowns In this assay, 100 μg of DOCA and 1 0 μg of aldosterone have approximately equivalent activities Using three groups of 3 rats each the lambda is 0.23 (Lambda = standard deviation ÷ slope) Compounds B and F, in doses up to 1 500 μg, do not cause sodium retention nor interfere with the sodium-retaining activity of DOCA or aldosterone in this assay In our hands, normal humans on unselected diet excrete 1 to 4 micrograms per 24 hours

Active urine extracts were chromatographed on paper (12) The region of aldosterone was eluted and reassayed. With the larger amounts present in human urine, activity has been consistently confirmed in the eluate, and fluorescence in ultraviolet light and reduction with blue tetrazolium also gave results consistent with aldosterone The active, crude extracts of urine consistently have been found to possess activity indistinguishable from pure aldosterone. Other groups have reported similar results (13)

Figure 1 illustrates the effect of orally administered KCl on the concentration of serum potassium in normal and sodium-depleted dogs In four control dogs receiving 2 gm of NaCl daily, the serum K was not appreciably

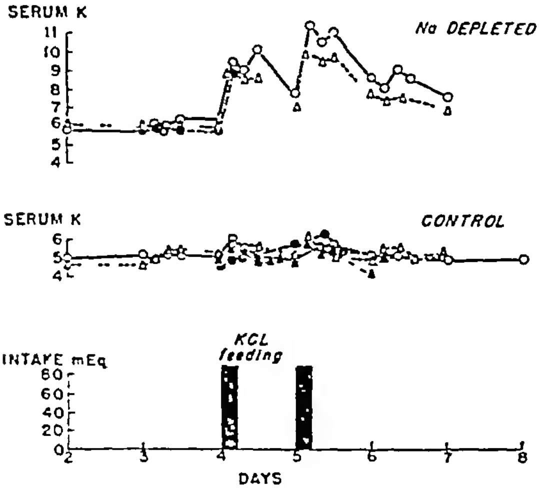

FIG. 1 HYPERKALEMIA RELATED TO SODIUM DEPLETION

Dogs rendered hyponatremic by peritoneal dialysis develop sustained hyperkalemia after feeding KCl. The same dose of KCl does not increase the serum potassium of animals not sodium depleted.

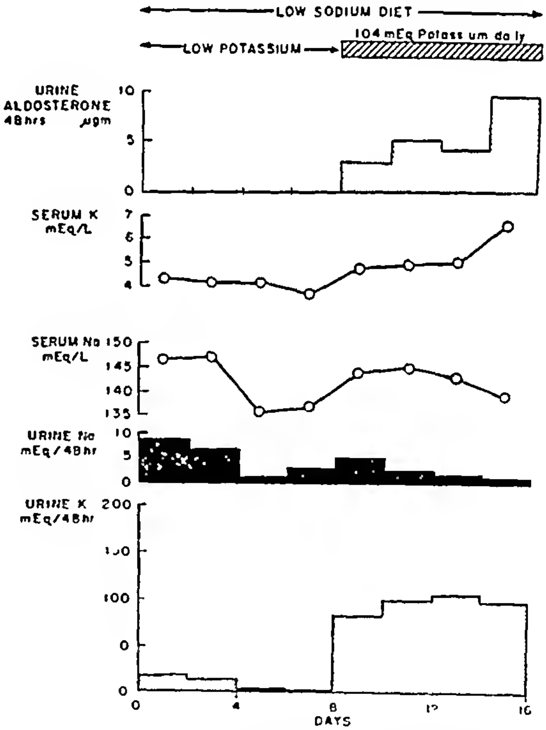

FIG 2. EFFECT OF LOW AND HIGH POTASSIUM INTAKE UPON ALDOSTERONE, SODIUM AND POTASSIUM EXCRETION IN DOG N ON A LOW SODIUM DIET

No detectable sodium retaining activity of urinary extracts was observed with a diet virtually free of sodium and potassium When dietary KCl supplements were given hyperkalemia developed and aldosterone-like activity appeared in the urine. No concurrent change in Na balance or serum Na was observed.

TABLE I

Feeding experiment in normal dogs

Dog	Experimental period	Consecutive days of balance period*	Daily intake Na (mEq)	Daily intake K (mEq)	Daily intake H_2O† (ml)	Observed serum‡ Na (mEq./L.)	Observed serum‡ K (mEq./L.)	Aldosterone activity 48 hr (Est. µg.)	Average daily urinary excretion§ Na (mEq)	Average daily urinary excretion§ K (mEq)	Average daily urinary excretion§ Vol. (ml.)
N	Control diet	7 8	51	104	450	147	4.2	0	44	94	400
	Na and K free diet	1 2	0	0	450	147	4.3	0	8.5	16.2	550
		3 4	↓	↓	↓	148	4.2	0	6.1	10.6	470
		5 6	↓	↓	↓	136	4.2	0	1.8	3.0	480
		7 8	↓	↓	↓	137	3.8	0	2.2	1.8	525
	KCl added daily from day 9	9 10	0	104	450	143	4.9	2.8	4.7	82.0	405
		11 12	↓	↓	↓	145	5.1	5.2	2.1	98.0	415
		13 14	↓	↓	↓	142	5.3	4.6	1.7	103.0	425
		15 16	↓	↓	↓	139	6.7	9.0	1.2	92.0	410
W	Control diet (KCl added) on day 7 8	5 6	51	80	450	146	4.4	0	40	77	485
		7 8	51	230	450	151	4.9	0	37	221	590
	Na free	1 2	0	67	450	143	4.8	0	18	74	555
		3 4	↓	67	↓	145	4.5	0	5.1	54	380
		5 6	↓	104	↓	147	4.7	0.5	4.4	113	367
		7 8	↓	104	↓	148	5.8	0.7	3.6	86	375
	Na and K free diet	9 10	↓	0	↓	145	4.6	0	0.5	16.8	510
		11 12	↓	↓	↓	144	3.6	0	0.4	2.4	455
		13 14	↓	↓	↓	144	4.3	0	1.8	0.9	480
		15 16	↓	↓	↓	143	3.8		0.5	0.7	478

* Refers to days from beginning of the particular diet
† Distilled water given daily by stomach tube throughout.
‡ Refers to serum levels obtained in fasting state at end of the corresponding 48-hour balance period
§ The average of two consecutive 24-hour measurements.

increased by KCl feeding In contrast, dogs previously depleted of Na by peritoneal dialysis, so that the serum sodium was reduced to about 120 mEq per L. uniformly developed a marked hyperkalemia when given the same amounts of KCl Two dogs (M and R) died after receiving a single oral dose of 10 gm. of KCl when severely depleted of sodium These dogs had tolerated even higher doses of KCl when on normal sodium intakes. This phenomenon has been utilized as a means of producing relatively sustained hyperkalemia.

The present studies were performed on 1) normal dogs 2) dogs with diabetes insipidus and 3) a single human subject with chronic congestive heart failure and edema.

1) Normal dogs

Three types of experiments were performed on these animals

(*a*) *Feeding experiment* (*Table I Figure 2*) One of two dogs (N) received a constant intake of a sodium and potassium free diet for 8 days, and in the succeeding 8-day period KCl was added. In the other dog (W) this procedure was reversed, KCl being added in the first and absent in the second experimental period. Distilled water (450 ml.) was given daily by stomach tube.

(*b*) *Depletion experiment* (*Table II Figure 3*) The dogs were first depleted of sodium chloride by peritoneal dialysis according to the method of Darrow and Yannet (14) and then maintained on a constant diet free of sodium. Repeated dialysis was necessary in several instances to achieve significant hyponatremia. Dogs R and M received 18 mEq of KCl on this diet whereas dogs W and E were kept on a K free as well as a Na free regimen. Distilled water (400 to 500 ml.) was given daily and, after a control period, KCl was added for two successive days by stomach tube.

(*c*) *Dilution experiment* (*Table III*) The serum sodium and potassium were reduced by a combination of forced hydration (1000 to 1500 ml. water by tube daily) and 2.5 units of Pitressin Tannate in Oil® injected twice daily Sustained hemodilution and hypervolemia can be achieved in this way with reduction in serum sodium and potassium (15)

2) Dogs with diabetes insipidus (D.I.) (Table II.)

Diabetes insipidus was produced by electrocoagulation of the hypothalamic tracts by Dr R. C. deBodo of the Department of Pharmacology New York University College of Medicine These dogs excreted from two to six liters of urine per 24 hours when having free access to food and water They were studied during three successive periods 1) unselected or stock diet, 2) sodium and potassium free diet, and 3) with the addition of KCl

extract was first dissolved in 4 ml of 50 per cent ethanol and dilutions of 1 16 and 1 80 were made.

The bioassay was carried out on male rats adrenalectomized 24 hours before the test. After adrenalectomy the rats were offered distilled water ad libitum and a low sodium diet (Na 0 001 per cent, K 0.3 per cent) At the start of the assay 5 ml of normal saline were injected intraperitoneally, and the bladder was emptied by electric shock. The animals were injected subcutaneously with either desoxycorticosterone acetate (DOCA) standards or unknowns in 0.25 ml of 30 per cent ethanol, and placed in groups of three rats in metabolism cages Duplicate groups of three rats were used for each DOCA standard (2, 10 and 50 micrograms) and for each unknown sample in 1 16 and 1 80 dilution. Human urine samples were handled similarly except that 24-hour collections were used, and extracts were assayed at 1 16, 1 80 and 1 400 dilutions When samples were strongly active at all dilutions throughout the range tested, appropriate additional five-fold dilutions were employed until a suitable end-point could be obtained. Further dilutions of inactive samples were tested since, with excessive amounts of aldosterone (near or greater than 10 micrograms per rat), sodium retention may not occur with this procedure.

At the end of a five-hour collection period the rats were forced to void by electric shock, the total urine volume was measured (4 to 10 ml per group), and the amount of sodium excreted determined by flame photometry The amounts of sodium excreted by the groups injected with DOCA were plotted on an arithmetic scale against the logarithm of the dose of DOCA (2, 10, 50 µg) The standard curve thus obtained serves for estimation of the sodium-retaining equivalents of the unknowns In this assay, 100 µg of DOCA and 1 0 µg of aldosterone have approximately equivalent activities Using three groups of 3 rats each, the lambda is 0.23 (Lambda = standard deviation − slope) Compounds B and F, in doses up to 1,500 µg., do not cause sodium retention nor interfere with the sodium-retaining activity of DOCA or aldosterone in this assay In our hands, normal humans on un selected diet excrete 1 to 4 micrograms per 24 hours

Active urine extracts were chromatographed on paper (12) The region of aldosterone was eluted and reassayed. With the larger amounts present in human urine, activity has been consistently confirmed in the eluate, and fluorescence in ultraviolet light and reduction with blue tetrazolium also gave results consistent with aldosterone The active, crude extracts of urine consistently have been found to possess activity indistinguishable from pure aldosterone. Other groups have reported similar results (13)

Figure 1 illustrates the effect of orally administered KCl on the concentration of serum potassium in normal and sodium-depleted dogs In four control dogs receiving 2 gm of NaCl daily the serum K was not appreciably

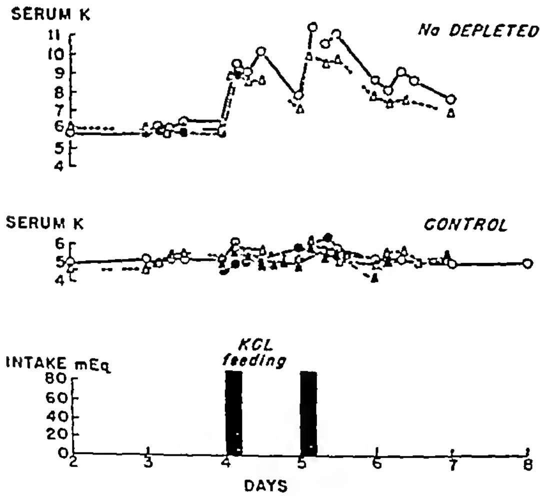

FIG 1 HYPERKALEMIA RELATED TO SODIUM DEPLETION

Dogs rendered hyponatremic by peritoneal dialysis develop sustained hyperkalemia after feeding KCl. The same dose of KCl does not increase the serum potassium of animals not sodium depleted.

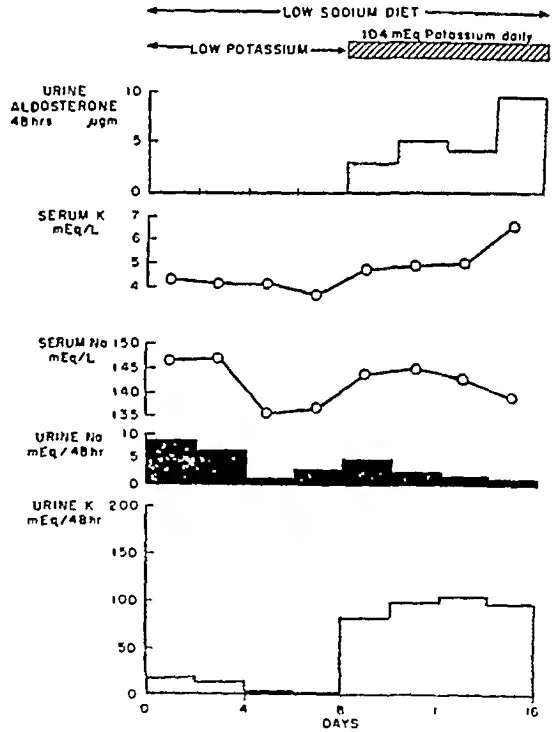

FIG 2 EFFECT OF LOW AND HIGH POTASSIUM INTAKE UPON ALDOSTERONE, SODIUM AND POTASSIUM EXCRETION IN DOG N ON A LOW SODIUM DIET

No detectable sodium-retaining activity of urinary extracts was observed with a diet virtually free of sodium and potassium. When dietary KCl supplements were given, hyperkalemia developed and aldosterone-like activity appeared in the urine. No concurrent change in Na balance or serum Na was observed.

TABLE I

Feeding experiment in normal dogs

Dog	Experimental period	Consecutive days of balance period*	Daily intake Na (mEq)	Daily intake K (mEq)	Daily intake H_2O† (ml)	Observed serum‡ Na (mEq./L.)	Observed serum‡ K (mEq./L.)	Aldosterone activity 48 hr (Est. μg)	Average daily urinary excretion§ Na (mEq)	Average daily urinary excretion§ K (mEq)	Average daily urinary excretion§ Vol. (ml)
N	Control diet	7 8	51	104	450	147	4.2	0	44	94	400
	Na and K free diet	1 2	0	0	450	147	4 3	0	8.5	16 2	550
		3 4	↓	↓	↓	148	4 2	0	6 1	10 6	470
		5 6	↓	↓	↓	136	4.2	0	1.8	3 0	480
		7 8	↓	↓	↓	137	3 8	0	2.2	1 8	525
	KCl added daily from day 9	9 10	0	104	450	143	4 9	2.8	4 7	82.0	405
		11 12	↓	↓	↓	145	5 1	5.2	2 1	98 0	415
		13 14	↓	↓	↓	142	5.3	4 6	1 7	103 0	425
		15 16	↓	↓	↓	139	6 7	9 0	1.2	92 0	410
W	Control diet (KCl added) on day 7 8	5 6	51	80	450	146	4 4	0	40	77	485
		7 8	51	230	450	151	4 9	0	37	221	590
	Na free	1 2	0	67	450	143	4 8	0	18	74	555
		3 4	↓	67	↓	145	4 5	0	5 1	54	380
		5 6	↓	104	↓	147	4 7	0 5	4 4	113	367
		7 8	↓	104	↓	148	5 8	0 7	3 6	86	375
	Na and K free diet	9 10	↓	0	↓	145	4 6	0	0.5	16 8	510
		11 12	↓	↓	↓	144	3 6	0	0 4	2 4	455
		13 14	↓	↓	↓	144	4.3	0	1 8	0 9	480
		15 16	↓	↓	↓	143	3 8		0.5	0 7	478

* Refers to days from beginning of the particular diet
† Distilled water given daily by stomach tube throughout.
‡ Refers to serum levels obtained in fasting state at end of the corresponding 48-hour balance period
§ The average of two consecutive 24-hour measurements.

increased by KCl feeding In contrast, dogs previously depleted of Na by peritoneal dialysis, so that the serum sodium was reduced to about 120 mEq per L., uniformly developed a marked hyperkalemia when given the same amounts of KCl. Two dogs (M and R) died after receiving a single oral dose of 10 gm. of KCl when severely depleted of sodium. These dogs had tolerated even higher doses of KCl when on normal sodium intakes Thus phenomenon has been utilized as a means of producing relatively sustained hyperkalemia.

The present studies were performed on 1) normal dogs, 2) dogs with diabetes insipidus, and 3) a single human subject with chronic congestive heart failure and edema.

1) *Normal dogs*

Three types of experiments were performed on these animals

(*a*) *Feeding experiment* (*Table I Figure 2*) One of two dogs (N) received a constant intake of a sodium and potassium free diet for 8 days and in the succeeding 8-day period KCl was added. In the other dog (W) this procedure was reversed, KCl being added in the first and absent in the second experimental period. Distilled water (450 ml) was given daily by stomach tube.

(*b*) *Depletion experiment* (*Table II Figure 3*) The dogs were first depleted of sodium chloride by peritoneal dialysis according to the method of Darrow and Yannet (14) and then maintained on a constant diet free of sodium Repeated dialysis was necessary in several instances to achieve significant hyponatremia. Dogs R and M received 18 mEq. of KCl on this diet whereas dogs W and E were kept on a K free as well as a Na free regimen. Distilled water (400 to 500 ml.) was given daily and, after a control period, KCl was added for two successive days by stomach tube.

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Diabetes insipidus was produced by electrocoagulation of the hypothalamic tracts by Dr R. C. deBodo of the Department of Pharmacology New York University College of Medicine. These dogs excreted from two to six liters of urine per 24 hours when having free access to food and water They were studied during three successive periods 1) unselected or stock diet, 2) sodium and potassium free diet, and 3) with the addition of KCl

TABLE II

NaCl depletion experiments in normal dogs

Dog	Experimental period	Consecutive days of balance period	Daily intake Na (mEq)	Daily intake K* (mEq)	Daily intake H_2O† (ml)	Observed serum Na (mEq/L.)	Observed serum K (mEq/L.)	Aldosterone activity 48 hr (Est. μg)	Average daily urinary excretion Na (mEq)	Average daily urinary excretion K (mEq)	Average daily urinary excretion H_2O (ml)
R	Control diet	3, 4	51	107	500	148	4 0	0	46	101	525
	Balance study started 8 days after a dialysis	1, 2	0	18	500	128	4 2	1 5	0 5	8 8	460
		3, 4		105		137	6 9	11 2	2 3	85 0	560
		5, 6		18		130	6 2	16 8	1 3	27 5	435
		7, 8	↓	18	↓	129	4.5	3 6	0 7	14 2	490
	A second study started 6 days after another dialysis	1, 2	0	18	500	139	5 1	6 8	0 3	11 7	482
		3, 4		105		141	7 1	11 4	1 6	99 5	570
		5, 6		18		138	5 9	4 8	0 5	19 1	480
		7, 8		18		137	5 0	2 0	0 4+	13 5+	415+
		9, 10	↓	18	↓	137	4 8	0 8	0 4	11 7	445
M	Study started 7 days after dialysis	1, 2	0	18	400	134	5 8	5 1	0 4	15 4	265
		3, 4		132		131	6 8	9 6	2 2	107 0	355
		5, 6	↓	18	↓	129	5 0	5 0	0 4	27 0	322
W	Stock diet	1, 2			500	143	4 5	0	105	62	550
	Study started the day after a dialysis Na and K free diet	1, 2	0	0	500				0 5	15 1	325
		3, 4				134	4.5	0 5	0 3	4 5	410
		5, 6				133	4 3	0	0 3	2 9	480
		7, 8		↓		132	4 4	0 8	0 3	2 1	462
		9, 10		94		132	5 5	0 5	6 5	86 6	462
		11, 12	↓	0	↓	133	4 7	0 5	0 2	9 4	540
C	Stock diet	1, 2			500	145	4 2	0	42 4	46 3	412
	Balance study started 1 day after a dialysis	1, 2	0	0	500				0 8	27 4	490
		3, 4				123	4 6	1 1	0 5	20 0	455
		5, 6				125	5 2		0 7	14 0	525
		7, 8		↓		124	5 3	5 9	0 8	22 4	525
		9, 10		67		135	6 0	9 6	3 3	56 4	530
		11, 12	↓	0	↓	127	5 4	2 2	2 6	15 4	495

* K was given as KCl by stomach tube as a 20 per cent solution The figures given are the averages of two doses for each two-day balance period

† Represents total daily free water intake and does not include water used in mixing diet batches

in amounts similar to those used in the normal dogs Distilled water was allowed ad libitum in all periods

3) A patient (Figure 5) with rheumatic heart disease

The patient was admitted to the metabolism ward and studied while on a constant regimen He was a 52 year-old man with mitral insufficiency, auricular fibrillation and chronic, right-sided congestive failure. He had taken digitalis preparations daily for fifteen years and Mercuhydrin® injections, as often as twice a week, for over ten years He had resorted to a low-sodium diet for at least ten years Maintenance digitoxin was continued in the hospital The sodium intake was kept constant throughout the study (12 mEq daily) The rates of aldosterone excretion on a low (16 mEq per day) and relatively high (140 mEq per day) potassium intake were compared.

RESULTS

The results of the animal studies are summarized in the accompanying tables and in Figures 1–4

Feeding experiments

In the experiments with two normal dogs (N and W, Table I) and two dogs with diabetes insipidus (T and E, Table IV), it was not possible to detect any sodium-retaining activity in the urine during periods of stock diet intake, or when KCl was given without simultaneous sodium deprivation The KCl supplements ranged from 67 to 230 mEq per day

Dog N was kept on a diet free of sodium and potassium for a period of 8 days During this

TABLE III

Dilution experiments in normal dogs

Dog	Experimental period	Consecutive days of balance period	Daily intake Na (mEq)	Daily intake K (mEq)	Daily intake H_2O* (ml.)	Observed serum Na (mEq/L.)	Observed serum K (mEq/L.)	Aldosterone activity 48 hr (Est. μg)	Average daily urinary excretion Na (mEq)	Average daily urinary excretion K (mEq)	Average daily urinary excretion H_2O (ml.)
N	Pitressin® 2.5 units b.i.d on days 5 6 7 8	3 4	0	0	ad lib	142	4 4		5 0	4.8	380
		5 6	0	0	1 125	122	3.6	0	8 1	3.5	615
		7 8	0	204†	900	129	6 2	2 6	24.5	190.9	578
W	Pitressin in Oil® 2.5 units b.i.d. on days 5 6	3 4	0	0	ad lib	143	4.5	0	5 9	10 7	390
		5 6	0	0	1,225	128	3 6	0	10 9	11 6	460

* Distilled water given by stomach tube daily or ad libitum as indicated
† Dog vomited 200 ml and this amount of K was subtracted from balance.

time no sodium-retaining activity was detected in the urinary extracts When 104 mEq of potassium were added to the diet significant hyperkalemia developed and salt retaining activity appeared in appreciable amounts in the urine (Figure 2)

In a second normal dog (W) the administration of KCl did not produce as great an elevation of serum potassium and insignificant amounts of sodium retaining activity were detected in the urinary extracts

Table IV presents data from similar feeding experiments in two dogs with diabetes insipidus These animals also as stated above appear to excrete little or no aldosterone-like material when on a stock diet or on a diet free of sodium and potassium When potassium chloride was added to the diet an increase in the sodium retaining activity of the urinary extracts was observed in both dogs Water balance was not detectably altered and sodium balance did not change appreciably A consistently low urinary specific gravity throughout the experiment affords further evidence that hydration was adequately maintained In the dogs with diabetes insipidus the serum potassium concentration was maintained at slightly higher levels than in the normal dogs during both the control and experimental periods.

Depletion experiments

Table II summarizes five balance studies in dogs depleted of sodium by peritoneal dialysis In three of the four animals detectable amounts of salt retaining hormone appeared in the urine at some time during the study The amounts excreted did not correlate well with the degree of hyponatremia, nor were they related to a further loss of body sodium or water but appear to be more directly related to the level of the serum potassium. It is noteworthy that the largest amounts of hormone were generally excreted on the days when potassium intake was greatest (R, M, C)

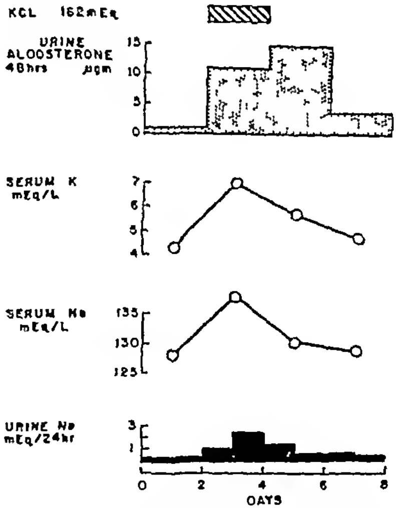

FIG. 3 EFFECT OF POTASSIUM FEEDING ON ALDOSTERONE EXCRETION AND SERUM POTASSIUM DURING SODIUM DEPLETION IN DOG R

Demonstration that sodium depletion with hyponatremia did not increase urinary aldosterone-like activity until dietary potassium was increased. No change in over all sodium balance was observed.

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PHYSICAL BINDING OF INSULIN BY GAMMA GLOBULINS OF INSULIN-RESISTANT SUBJECTS[1,2]

By BELTON A. BURROWS, THEODORE PETERS[3] and FRANCIS C. LOWELL with the technical assistance of ANNE N. TRAKAS and PAUL REILLY

(From the Radioisotope Service, Boston Veterans Administration Hospital, the Robert Dawson Evans Memorial, Massachusetts Memorial Hospitals, the Department of Medicine, Boston University School of Medicine, and the Department of Biological Chemistry, Harvard Medical School, Boston, Mass.)

(Submitted for publication July 2, 1956; accepted November 15, 1956)

The occasional occurrence of resistance to insulin among certain diabetic patients has been recognized for many years. However, the immunologic nature of this resistance, which may require the administration of over 1,000 units of insulin per day, has been demonstrated only by indirect means (2). Serum of insulin resistant patients has been shown to protect mice from the hypoglycemic effect of insulin (3, 4) and to prevent the *in vitro* action of insulin on the rat diaphragm (5). The insulin neutralizing property of such serum has been shown by electrophoretic (6, 7) and salt (8) fractionation to reside in the gamma globulin fraction. The substances in the plasma, presumably antibodies, related to the capacity of insulin to produce urticaria and allied reactions are apparently distinct from the insulin neutralizing substances and have been shown to reside in the beta globulin fraction (6).

Using I^{131} labeled insulin, this study has shown that serum of insulin resistant subjects, but not of normal individuals or non-resistant diabetic patients, complexes insulin and that this complex migrates at the leading edge of the gamma globulin fraction upon paper electrophoresis. By adding progressively larger amounts of insulin it was possible, in most cases, to estimate the concentration of insulin needed to saturate such sera. Seventeen serum specimens from four insulin resistant persons and one insulin resistant rabbit were found to bind insulin in amounts from 0.05 μgm. to more than 20 μgm. per ml.

MATERIALS AND METHODS

Preparation of labeled insulin. Insulin labeled with I^{131} and containing 3 μc. of I^{131} per μgm. and about 0.6 atom of iodine per 24,000 molecular weight was prepared by a modification of the basic method of Banks, Seligman, and Fine (9). A mixture was made of 0.15 ml. of KI containing 15 μgm. I with 0.02 ml. of KIO containing 3 μgm. of I in a 2 ml. glass stoppered tube. To this was added KI^{131} containing 14.8 μc. and about 0.12 μgm. of I followed by 0.1 ml. of 1 N HCl. After the addition of CCl the contents were shaken periodically for 10 to 15 min. during which time about 80 per cent of the I was extracted as I_2 into the CCl layer. The upper (aqueous) layer was removed by a dropper and 0.1 ml. of 0.12 N Na CO containing 1 mg. crystalline bovine insulin[4] was added. The tube was shaken and allowed to stand for one hour.

Unreacted iodine was removed by treatment with an anion exchange resin. The insulin solution was diluted with 0.4 ml. of pH 7.4 buffer containing 0.11 N NaCl and 0.04 M veronal and placed on a 0.8 × 12 cm. column of Amberlite IRA 410 which had been saturated with HCl and adjusted to pH 7.4 prior to use. The insulin was washed through the column with the buffer and finally made to 10.0 ml. with buffer. Yield was 20 per cent of the I^{131} used. Less than 4 per cent of the radioactivity of the insulin could be removed by prolonged dialysis. The I^{131} labeled insulin was used within a week of its preparation to minimize the possibility of radiation damage to the insulin. At concentrations less than 10 μgm. per ml. the insulin was found to adsorb appreciably onto glass containers so it was not diluted until just before use.

Testing procedure. After 0.02 ml. of labeled insulin solution, containing from 5 to 100 μgm. of insulin per ml. was added to 0.2 ml. of serum, 0.02 ml. of this mixture, or 0.2 μgm. of insulin, was applied in a narrow band across a 3-cm. strip of Whatman No. 3MM paper in the electrophoresis apparatus. A similar volume of the mix

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[1] Supported by AEC Contract At (30-1) 919 between the Atomic Energy Commission and the Massachusetts Memorial Hospitals.

[2] This work has appeared in abstract form (1) and was presented at the New England V.A. Clinical Research Society Meeting, Sturbridge, Massachusetts, October 7, 1955.

[3] Present address: The Mary Imogene Bassett Hospital, Cooperstown, New York.

[4] Armour's 5 × recrystallized bovine insulin, graciously supplied by Dr. E. G. Ball of the Department of Biological Chemistry, Harvard Medical School.

ture was delivered into 1 ml of water for subsequent determination of the exact amount of I^{131}-insulin present by gamma ray counting The paper strips had been previously wet with the buffer (veronal pH 8 6 ionic strength 0 05) and allowed to equilibrate for 20 to 40 minutes in the apparatus The electrophoresis equipment was of a conventional type employing horizontally suspended strips, with a free length of 38 cm between electrode baths About 5 v per cm of free length were applied for 14 to 18 hours at room temperature The strips were then dried in an oven at 95° C for 30 minutes

RESULTS

In Figure 1 are shown typical results on different types of sera Insulin labeled with radioiodine was detected by exposing the strips against X-ray film for 1 to 8 days Proteins were subsequently detected by staining with 0 25 per cent bromphenol blue in 9/1 methanol/acetic acid and rinsing in 30, 3/1 water/acetic acid/phenol

The findings agreed with those of Kallee (10, 11) in that insulin in these low concentrations, whether tested alone or mixed with normal serum, did not migrate upon electrophoresis, but remained adsorbed to the paper at the starting point (Figure 1, strips 1 and 2) Migration of free insulin could be obtained by adding a large excess of insulin also shown by Kallee In these cases the I^{131}-insulin migrated with a mobility about equal to that of serum albumin, but exchange between the labeled and non-labeled insulin and adsorption along the strips resulted in smearing and trailing of the labeled insulin Hence, the more precise test for binding of insulin was to examine strips for movement of insulin which otherwise would have remained adsorbed at the starting point

Strips 3, 4 and 5 of Figure 1 show binding of the I^{131}-insulin by the sera of three patients who were resistant to insulin In strips 3 and 4 the insulin is completely bound so that none remains at the starting point In strip 5 the serum has apparently been saturated, so that only a portion of the insulin moves Insulin which is bound moves with the leading portion of the gamma globulins suggesting that the insulin is complexed by gamma globulins into a soluble molecule with a mobility intermediate between the mobilities of free insulin and free gamma globulin

In the detailed studies of Singer and Campbell (12, 13) on the physical chemical properties of soluble antigen-antibody complexes of ovalbumin and bovine serum albumin with their rabbit antibodies it was shown that the electrophoretic mobilities of complexes were intermediate between

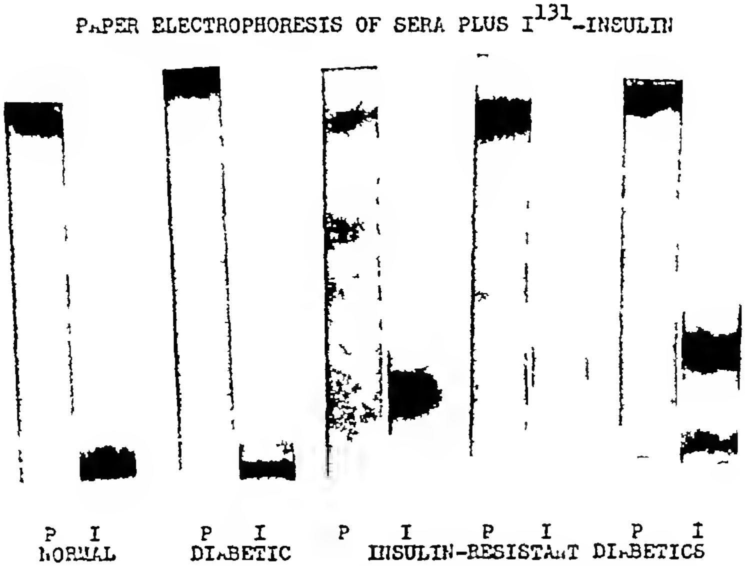

FIG 1 ELECTROPHORESIS OF I^{131} INSULIN MIXED WITH VARIOUS SERA
Strips labeled P are stained to show the protein fractions Radioautographs of the same strips are labeled I

the mobilities of the free antigen and antibody and could be predicted on the basis of the weight fraction of each component in the complex From rough measurements of the relative mobilities on paper of free insulin (on insulin treated papers) gamma globulin and complexed I^{131} insulin, it can be calculated on this basis that insulin comprises only 7 to 13 per cent of the weight of the complex Assuming a molecular weight of 170-000 for the gamma globulin this would mean that the complex contains one-half to one insulin molecule of molecular weight 24 000 per one gamma globulin molecule

Quantitative measurements

For routine testing radioautographs were not made but the labeled insulin was located by passing the strip under a 1 9 mg per sq cm end window Geiger counter equipped with a lead cover containing a 0 5-cm slit Radioactivity was measured every 0 5 or 1 cm of length along the paper strip and the curve of radioactivity versus distance along the strip plotted Movement of the labeled insulin could be detected by comparing the curve with the strip after staining to locate the protein fractions When only part of the insulin migrated the amount which migrated was estimated by determining the areas under the two curves with a planimeter

In varying the insulin concentration to determine the saturation limit of the sera the insulin concentration was not allowed to exceed 10 μgm per ml of serum To avoid exceeding this level the sera under investigation was diluted with varying amounts of serum from normal individuals This also avoided the possibility of exceeding the saturation limit of the paper when insulin would migrate in the free form

Table I shows the results with various types of subjects Eight normal persons and seven diabetic patients who were not resistant to insulin showed no migration of I^{131} insulin with the gamma globulins the level of detection being approximately 0 02 μgm or 0 0007 unit of insulin per ml serum The sera of four insulin resistant patients and of a rabbit immunized against crystalline beef and pork insulin by one of the authors in a previous study (14) showed binding of insulin in ranges of 0 4 to 20 μgm per ml Some of the specimens had been stored frozen with several thawings for periods up to 15 years but still gave satisfactory electrophoretic patterns

Occasional modifications of the response were noted, such as a suggestion of a peak trailing the serum proteins (Figure 1 strip 4) or tailing or movement of the baseline These modifications were seen occasionally with sera of resistant patients when large amounts of insulin were added and were never seen with normal sera or with sera of diabetic patients who were not resistant to insulin except in one specimen from a diabetic patient which showed a slightly increased tailing of the baseline These effects may represent partial binding by serum with weak forces of the same order as the adsorption affinity of insulin for the paper

With one patient it was possible to compare the amounts of insulin bound with the insulin requirement and the results of mouse protection tests on the same serum specimens (Table I) The periods of resistance to insulin as shown by the requirement for insulin and mouse test correspond with periods of high binding of insulin by the gamma globulins.

Species specificity

Table II shows evidence of species specificity of the insulin binding when tested by adding nonlabeled human insulin in addition to the labeled bovine insulin to sera from insulin resistant subjects The amount of binding of I^{131} bovine insulin which was observed was not reduced by the addition of human insulin to the degree expected if the two insulins were equally bound This preferential binding of I^{131} bovine insulin is in agreement with the finding (15 16) that human insulin was effective in controlling the blood sugar in patient A M when large doses of bovine insulin were ineffective and in lowering the blood sugar in Rabbit No 5 (14) when equivalent or larger doses of bovine insulin had no effect

Recent reports have indicated (17 18) that sera of insulin treated diabetic patients who showed no clinical resistance to insulin bound labeled insulin either *in vivo* or *in vitro* so that it migrated with or just ahead of the gamma globulins The present study has not demonstrated binding of insulin by gamma globulins in any of

TABLE I

I^{131}-insulin bound by serum proteins

Subject	Date	Clinical insulin resistance*	Insulin dose administered† Units/day	Type	Insulin neutralization by mouse test	Insulin bound by gamma glob (µgm/ml)
8 normals			0			0
7 diabetics			0–46	NPH		0§
A M	2/11/42	?0	0	Reg	0	0.05
	3/2/42	—	800+	Reg	+	10+
	8/18/42	?0	35	Reg	?	0.05
	11/4/42	0	35	Reg	0	0 (tailing)
	4/19/44	0	0–96‡	Reg	0	0 (tailing)
	5/25/44	+	280+	Reg	+	20
C	4/13/44	+	ca. 300	Reg		0.8+
	9/10/44	+	ca. 300	Reg		0 (tailing)
	12/17/44	+	ca. 300	Reg		1+
M	9/7/44	+	ca. 500	Reg		1.5
	9/21/44	+	ca. 500	Reg		1.2
	4/15/48	+	ca. 500	Reg		0.6+
	8/8/55	?	100	Reg		0.14
O	3/23/54	+	ca. 300	Reg		0.2
	4/24/54	+	ca. 300	Reg		0.4
Rabbit "No. 5"	9/26/47	+	(Immunized)	Cryst		0.2
Rabbit "No. 5" (14)	3/23/48	+	(Immunized)	Cryst		0.4

* Presence of clinical resistance indicated by "+", absence of resistance by "0". A "?" indicates that no direct test for resistance was done but because of considerations discussed elsewhere (2) resistance was probably present or absent as indicated.

† The amount of insulin given does not necessarily reflect the insulin requirement, in that the patient may not have been completely controlled by the insulin dose indicated.

‡ Insulin therapy resumed two days previously, 31 to 96 units per day.

§ One specimen showed slight tailing of the adsorbed band.

the seven diabetics who were not resistant to insulin. No obvious reason could be found for this difference in results but it might be related to 1) difference in sensitivity of detection of binding, 2) the use of bovine insulin in this study instead of the commercial mixture of beef and pork insulin or 3) lower antigenicity of the NPH-insulin received by all but one of the non-insulin-resistant diabetic patients reported here.

TABLE II

Preferential binding of I^{131} bovine insulin in the presence of human insulin

Subject	I^{131} bovine insulin Added (µgm/ml)	Bound (µgm/ml)	Human insulin added (µgm/ml)
O 3/23/54	0.48	0.24	0
	0.44	0.23	0.44
A M 5/25/44	9	9	0
	60	22	0
	4	3.1	35
Rabbit No. 5	0.48	0.15	0
	0.54	0.14	0
	0.44	0.11	0.44

The technique described offers a sensitive method of testing for the presence of non-precipitating antibodies. It is somewhat similar to the technique of precipitation of labeled antigen with ammonium sulfate employed for this purpose by Farr (19), but differs from the paper electrophoresis technique used by others (20) for the detection of antibodies against DNP-beta-lactoglobulin, which depended upon the complexed antigen being rendered immobile by antibodies, apparently precipitin in nature. The above technique may be of value in testing for antibodies to protein hormones other than insulin.

SUMMARY

1. When I^{131}-labeled bovine insulin was added *in vitro* to serum from insulin-resistant persons the insulin was found to migrate with the leading edge

of the gamma globulin zone upon paper electrophoresis Similar migration was not seen with serum from normal persons or diabetic patients who were not resistant to insulin This suggests that the insulin resistance was due to the presence of antibodies which bound insulin into an inactive but soluble complex

2 Insulin binding capacity amounted to 0 05 to more than 20 μgm per ml of serum Evidence that human insulin was not bound in this manner is presented.

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PLASMA PROTEIN SYNTHESIS IN THE HUMAN FETUS AND PLACENTA[1]

By JOSEPH DANCIS,[2] NANCY BRAVERMAN, AND JOHN LIND

(From the Department of Pediatrics and of Obstetrics and Gynecology, New York University Bellevue Medical Center, New York, N. Y., the Wenner-Gren Cardiovascular Research Laboratory and the Department of Pharmacology, Royal Veterinary College, Stockholm, Sweden)

(Submitted for publication August 16, 1956; accepted November 16, 1956)

Electrophoretic studies of human fetal plasma have demonstrated a protein pattern in which all major fractions are present (1). The site of origin of these plasma proteins is not known. Presumably they may be synthesized by the mother and merely circulate in the fetus as the result of placental transfer, or they may be synthesized by the placenta or the fetus.

The gamma globulin level is high at birth and falls during the first weeks of life (2). This led to the assumption that the gamma globulin found at birth is derived from the mother by transfer through the placenta, and that the newborn infant does not synthesize it. Because gamma globulin levels (2) and antibody levels (3) are often higher in cord blood than in maternal blood there has been speculation that the placenta may be the site of synthesis. The problem of plasma protein synthesis in the placenta has been approached by comparing levels of the various fractions in blood from the umbilical artery and vein and from the mother (4). It was concluded that the placenta synthesizes albumin.

The present experiments were designed to investigate the synthesis of plasma protein by placenta and fetal liver. Term placenta was obtained at normal deliveries. Liver and placental tissues were also taken from three fetuses of 3 to 4 months gestation (Table I). For purposes of comparison fetal heart was similarly investigated. The fetuses were delivered surgically for psychiatric and social indications and were presumably normal. Tissue slices were incubated with glycine-2-C_{14} and the incorporation of radioactivity into plasma proteins was studied. The proteins were isolated and identified electrophoretically and by immunological techniques.

PROCEDURE

Incubation

Tissues were procured immediately after delivery and dropped into chilled isotonic saline. Slices about 0.5 mm thick were prepared with a Stadie-Riggs slicer. One gram of tissue was added to 2.5 ml. of Krebs-Ringer-bicarbonate buffer (5) in a 25 ml. flask to which had been added 5 micrograms of Aureomycin and about one million counts per minute of glycine-2-C_{14} (Tracerlab). Radioactivity was determined as described below. The slices were incubated with constant shaking for 18 hours in a water bath at 37° C.

Electrophoresis

At the end of incubation, the tissue slices were centrifuged down and discarded. Incubation media from each type of tissue were pooled and dialyzed against three changes of isotonic saline during 24 hours at 5° C. The bag contents were lyophilized and redissolved in 1 ml. isotonic saline to reduce to volumes suitable for electrophoresis. The samples were dialyzed for three hours against veronal buffer, pH 8.6, and then separated by starch electrophoresis and the protein pattern developed as described by Kunkel and Slater (6). One ml. of human plasma was run in parallel on the same block to serve as marker for the plasma protein fractions.

To determine the radioactivity curve, the protein was precipitated from an aliquot of each eluted fraction by the addition of equal volumes of 10 per cent trichloracetic acid (TCA). The precipitate was washed with 5 per cent TCA and extracted with ether to remove the TCA. The precipitate was redissolved in 0.05 N NaOH, transferred to a planchette, and the radioactivity determined.

Immunological identification of radioactive proteins

Liver. Carrier precipitates were made in eluates from the starch electrophoresis by adding various plasma fractions and their specific antisera prepared in rabbits. Fractions 5 to 12 (Figure 1), representing the alpha and beta globulins, were pooled, divided into five aliquots and

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TABLE I

Data on fetuses

Fetus	Weight (grams)	Gestation in days (history)	Gestation† in days (from weight)
I	150	130	120
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* Calculated from menstrual history

† Estimated from weight according to graph by Widdas (18)

a specific precipitate of about 2 mg was made in each aliquot. A preliminary titration was done in a small sample of each aliquot, so that appropriate amounts of antigen and antibody were added to produce the precipitate at or near the equivalence point. Precipitates were prepared with the following fractions human albumin, guinea pig albumin human beta globulin beta lipoprotein and metal-combining globulin. Similar precipitates were prepared in the albumin fraction (17 and 18, Figure 1) All of the specific precipitates were made in aliquots of the eluates containing approximately the same amount of radioactivity This permits comparison of the amount of radioactivity incorporated in each specific precipitate.

The fibrinogen was located in fractions 12 to 14 by adding thrombin to the concurrently separated human plasma. A precipitate was then formed in fractions 12 to 14 of the incubating medium by adding carrier fibrinogen and the specific antiserum. A similar control precipitate was made in fractions 16 to 18.

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Placenta. After electrophoretic separation, it was demonstrated that the radioactive proteins were distributed in the alpha and beta globulin fractions. These fractions were separated (Figure 3 fractions 5 to 9 and 10 to 14 respectively) and specific precipitates were made in each fraction.

In earlier experiments with term placentas the technique described by Keston and Katchen was used (8) After incubation the tissues were homogenized and the mixture centrifuged. The supernatant was dialyzed against saline and specific precipitates were made in aliquots of the supernatant without preliminary electrophoresis. To provide an estimate of non specific adsorption by the protein precipitates specific precipitates were made

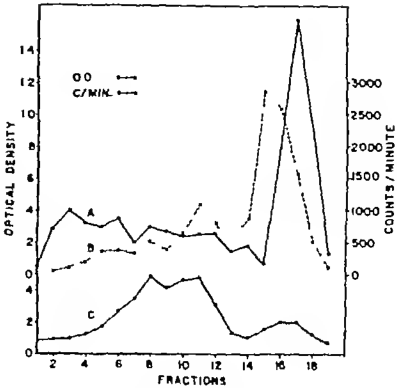

FIG. 1 SYNTHESIS OF PROTEINS BY FETAL LIVER

A Electrophoretic pattern of human plasma proteins

B Total radioactivity of proteins in the incubating medium. Note similarity to A.

C Pattern of proteins in incubation medium.

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Preparation of specific antisera

Antisera were prepared in rabbits by the injection of plasma protein fractions with Freund's adjuvant (9) Most of the antisera were prepared by injecting interdigitally 3 to 5 mg of antigen emulsified in 0.5 ml. Falba, 1 ml. Bayol F and 1 mg of killed tubercle bacilli. Some of the antisera prepared early in the study required 50 mg of antigen in 4 ml. of adjuvant, and the injections were intramuscular

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Placenta. After electrophoretic separation it was demonstrated that the radioactive proteins were distributed in the alpha and beta globulin fractions. These fractions were separated (Figure 3, fractions 5 to 9 and 10 to 14 respectively) and specific precipitates were made in each fraction.

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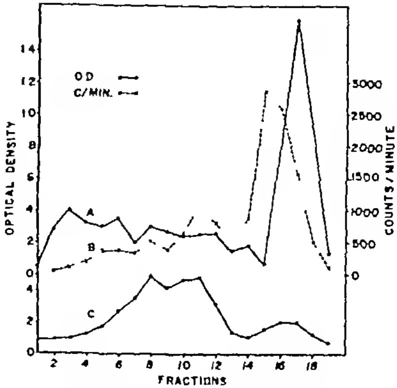

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The beta globulins were prepared by starch electro-

phoresis of human plasma. Antisera were prepared against alpha and beta globulin fractions individually but were found to be immunologically indistinguishable by the techniques used in this study. They were then used interchangeably. The guinea pig albumin and antiserum were prepared in similar fashion.

Determination of radioactivity

The protein precipitates were transferred in solution to aluminum planchettes covered with filter paper to produce even distribution and dried under an infra-red lamp. Radioactivity was determined in a D-46A Nuclear Flow Gas Counter, and the counts were corrected to infinite thinness.

RESULTS

The electrophoretic pattern of normal plasma proteins (Figure 1 A) is paralleled strikingly by the total radioactivity of the proteins in the medium used for the incubation of *liver* slices (Figure 1 B), except that there is no incorporation of radioactivity into proteins with gamma globulin mobility. The electrophoretic pattern of the proteins in the incubation medium (Figure 1 C), probably representing primarily liver proteins, is quite dissimilar.

Carrier precipitates were made by adding various plasma proteins and their specific antisera to fractions of the electrophoretically separated proteins, as previously described. The results are presented in Table II. It is evident that a significant amount of radioactivity is carried down by a specific precipitate prepared in the appropriate fraction (*e.g.*, the human albumin precipitate in the albumin fraction) and that it is considerably less in the inappropriate portions of the curve (*e.g.*, the albumin precipitate in the globulin fraction). Precipitates prepared with guinea pig albumin and its specific antiserum to give an index of non-specific adsorption, also contain much less radioactivity. The gamma globulin precipitates seemed to give non-specific results. This suspicion was verified by demonstrating extensive cross-reaction between human albumin and our gamma globulin antiserum. The gamma globulin precipitates also incorporated as much radioactivity as the beta globulin precipitates even though the radioactive proteins had an electrophoretic mobility corresponding to the latter.

The results of the experiments with *fetal heart* are presented in Figure 2 and Table III. The electrophoretic pattern of the radioactive proteins in the incubation medium (Figure 2 B) resembles more closely that of the presumed tissue proteins (C) than the plasma proteins (A). The amount of radioactivity incorporated is much less than in the liver experiment (note the difference in scale in Figures 1 and 2).

Unfortunately there was insufficient material after electrophoretic separation to permit immunological identification. Accordingly, in a second experiment, preliminary separation was done with ammonium sulfate. There is a significant amount of radioactivity in the beta globulin and albumin precipitates made in the supernatant (Table III). However, the results with guinea pig albumin in-

TABLE II

Incorporation of radioactivity into proteins by fetal liver (counts per minute) *

Precipitates	Experiment II		Experiment III	
	Globulin fraction	Albumin fraction	Globulin fraction	Albumin fraction
Total protein†	1594	1854	1210	915
Albumin (human)	95	1130	51	980
Beta globulin	670	44	598	13
Metal-combining	171	10	148	2
Lipoprotein	239	32	279	18
Gamma globulin	688	566	560	169
Albumin (guinea pig)	58	26	27	50
	Fibrinogen fraction	Fibrinogen control	Fibrinogen fraction	Fibrinogen control
Total protein†	574	890	474	572
Fibrinogen	186	78	201	105

* Carrier precipitates were formed by adding the protein and its specific antiserum to appropriate electrophoretic fractions (*e.g.* albumin precipitate in albumin fraction) and as a control, in inappropriate fractions (*e.g.* albumin in globulin fraction). Guinea pig albumin precipitates are further controls.

† TCA precipitable radioactivity.

TABLE III

Incorporation of radioactivity into specific precipitates by fetal heart (counts per minute) *

	Globulin fraction	Albumin fraction
Total protein	107	124
Albumin (human)	0	56
Beta globulin	10	40
Gamma globulin	8	
Albumin (guinea pig)		38

* Incubation medium proteins were separated by ammonium sulfate precipitation into two fractions and specific precipitates made in each fraction.

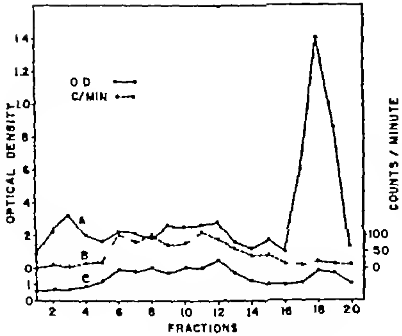

FIG 2. SYNTHESIS OF PROTEINS BY FETAL HEART
A Electrophoretic pattern of human plasma proteins
B Total radioactivity of proteins in the incubating medium.
C Pattern of proteins in incubation medium Note similarity of B and C in this figure.

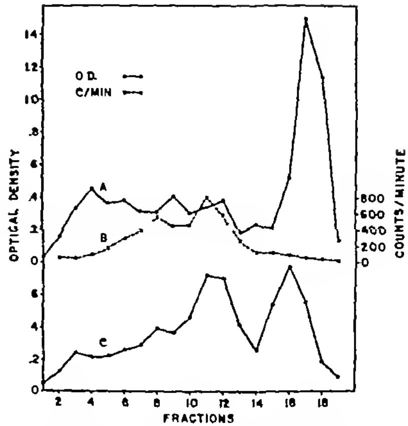

FIG 3 SYNTHESIS OF PROTEINS BY TERM PLACENTA
A Electrophoretic pattern of human plasma.
B Total radioactivity of proteins in the incubating medium.
C Pattern of proteins in incubation medium

dicate that this is probably because of non specific adsorption

The results of an experiment with term *placenta* are given in Figure 3 The total radioactivity of the proteins in the incubating medium (B) parallels the protein pattern (C) more closely than that of the plasma proteins (A) There is no radioactivity in proteins with albumin or gamma globulin mobilities It is presumed that the albumin peak in C is derived from blood in the tissue slices since the placenta apparently does not synthesize proteins with this mobility (B)

The results of similar experiments with placentas from 3 to 4-month pregnancies are not reproduced here because there are no important differences The radioactive proteins moved primarily in the beta and alpha globulin zones There were no radioactive proteins with albumin mobility but a small amount overlapped the faster moving gamma globulins As with term placenta the pattern resembled more closely that of the proteins of the incubating medium than that of plasma proteins

Carrier precipitates were made by adding plasma proteins and the specific antisera to the alpha and beta globulin fractions (Figure 1 fractions 5 to 9 and 10 to 14 respectively) In the early placenta the amount of radioactivity in the precipitates was about the same as that obtained with guinea pig albumin and probably represented non specific absorption However in term placentas there was significantly greater radioactivity in the precipitates prepared with plasma globulins (Table IV)

In earlier experiments using immunological isolation of proteins without preliminary electrophoretic separation (Table V) there is more radioactivity in the gamma globulin precipitate than in the control precipitates In the type of experiment presented in Table V A, the slices were homogenized in the incubating medium and the immunological precipitates made in the supernatant In Table V B the precipitates were made in the incubating medium without preliminary homogenization of the tissue. The reduction in non specific adsorption is evident

DISCUSSION

This investigation was originally undertaken to determine if the placenta could synthesize plasma proteins The albumin and gamma globulin fractions were first investigated because reports from other workers had suggested that the placenta may synthesize these fractions The

TABLE IV

Incorporation of radioactivity into proteins by placenta (counts per minute)

Protein	Early placenta*		Late placenta*	
	Beta globulins	Alpha globulins	Beta globulins	Alpha globulins
Total protein	213	260	368	443
Albumin (human)	7	7	0	25
Alpha globulin			30	105
Beta globulin	4	21	36	125
Gamma globulin	19	19	43	124
Albumin (egg)			8	2
Albumin (guinea pig)	18	14		

* Early placenta was from a 3 to 4 months gestation, late placenta was at term. Carrier precipitates were made by adding the plasma protein fraction and its specific antiserum to aliquots after electrophoretic separation. Total protein refers to TCA precipitable radioactivity.

TABLE V

Incorporation of radioactivity into specific precipitates by term placenta (counts per minute)

	A*	B†
Egg albumin	75	6
Human albumin	77	4
Bovine gamma globulin	115	30
Human gamma globulin	355	72

* A. The tissue slices were homogenized in the incubating medium and carrier precipitates prepared in the supernatant.

† B. Carrier precipitates were made in the incubating medium without preliminary homogenization of tissues.

technique described by Keston and Katchen (8) (see Procedure) seemed suitable, and in Table V A are presented the results of a typical experiment with term placenta. The amount of radioactivity incorporated into the gamma globulin precipitate appeared significant; however, the amount of radioactivity in the control precipitates was also appreciable. In a second series of experiments (Table V B), the specific precipitates were made in the incubating medium without preliminary homogenization of the tissue, since it seemed reasonable that the plasma proteins would diffuse out of the cell. This reduced the amount of radioactivity brought down in the control precipitates verifying the negative results with albumin and making more significant the results with gamma globulin. Cross-reaction between bovine and human gamma globulin (11) probably accounted for the intermediate amount of radioactivity in the former precipitates. These results have been previously presented (12).

The technique of electrophoretic separation followed by immunological precipitation was resorted to in an attempt to better identify the proteins. The data obtained with fetal liver and heart will be discussed first, since they add to the interpretation of the results with placenta.

The experiments with liver give clear cut evidence that the fetus is already capable of synthesizing plasma proteins by 3 to 4 months gestation. The electrophoretic separations indicate that the liver is capable of synthesizing all of the electrophoretically identifiable proteins except gamma globulin. These results conform with those of perfusion experiments with adult rat liver (13) and slice experiments with adult guinea pig liver (14). The immunological studies confirmed the identity of the proteins and gave further information about sub-fractions of the beta globulins.

The gamma globulin precipitates gave puzzling results. The radioactivity carried down in the albumin fraction was probably the result of cross-reaction. We later demonstrated extensive cross-reaction between our gamma globulin antiserum and albumin. However, the gamma globulin precipitates were just as effective as the β globulin precipitates in bringing down radioactivity among proteins with beta and alpha globulin mobility. This was also true in experiments with term placenta, as will be discussed below. It is well known that the plasma globulins are closely related immunologically (15). The question may be raised as to whether immunological differentiation is even less distinct at this early stage of development.

The results with fetal heart are clearly different from those obtained with liver, and conform with what might be expected from an organ that synthesizes proteins for its own use. The radioactive proteins that have diffused into the medium are small in amount and the electrophoretic pattern resembles that of the non-radioactive proteins in the medium, probably representing tissue proteins.

Because of the small amount of tissue available from fetal heart, and the low level of incorporation of radioactivity into proteins, there was insufficient material after electrophoretic separation to permit the preparation of immunological precipitates in the various fractions. In the second experiment, ammonium sulfate precipitation was employed to separate roughly the proteins. The albumin and beta globulin precipitates made in the supernatant

after ammonium sulfate precipitation had radioactivity. Since this fraction might be expected to contain the albumin and a large proportion of the beta globulins and since there was also radioactivity in these fractions as determined electrophoretically this finding might have been considered significant. However, a heterologous precipitate indicated that the radioactivity was incorporated by non specific adsorption. The proportion of radioactivity brought down with the guinea pig albumin precipitate in this experiment may be contrasted with that in the fetal liver (Tables II and III).

In the experiments with term placenta electrophoretic identification of the radioactive proteins demonstrated that the placenta did not synthesize proteins with either albumin or gamma globulin mobility (Figure 3 B). The proteins moved into the alpba and beta globulin area and appeared to be related immunologically to all three globulin fractions in that each carrier precipitate brought down approximately the same amount of radioactivity (Table IV).

Particular pains were taken with the gamma globulin precipitates because of the paradoxical results with electrophoretic separation. In a series of experiments different antigens and different antisera were used. In one experiment the gamma globulin antigen was prepared by precipitating diphtheria toxoid with immune human serum the precipitate being largely composed of the specific anti diphtheria gamma globulin. The results in all experiments were essentially the same.

It would be of interest to know if the globulins synthesized by the placenta have functional similarities to plasma globulins. Good and Zak have presented an interesting report (16) of a woman with agammaglobulinemia who was immunized repeatedly during pregnancy. Antibodies were detected in the mother in the last months of pregnancy and circumstantial evidence indicated that the placenta might be the source of the antibodies. Since some antibodies have electrophoretic mobilities similar to those of the radioactive proteins in our experiments with placenta (17) we made attempts to demonstrate antibody synthesis using the Keston and Katchen technique with placentas from mothers immunized against tetanus and diphtheria. The attempts were unsuccessful. This does not exclude the possibility that the placenta synthesizes antibodies in amounts too small to be detected by this technique or that it may synthesize antibodies under abnormal conditions (for example agammaglobulinemia in the mother).

It is not likely that under normal circumstances the placenta contributes significantly to the plasma proteins of the fetus. The placenta from pregnancies of 3 to 4 months duration does not synthesize any plasma proteins detectable by this technique (Table IV). By this stage of pregnancy the fetal liver is already actively synthesizing all plasma protein fractions except the gamma globulin. The gamma globulins as identified electrophoretically are probably supplied by the mother.

SUMMARY

1 Liver slices from human fetuses of 3 to 4 months gestation were incubated with glycine 2 C_{14}. The proteins were separated electrophoretically and immunologically and the incorporation of radioactivity was determined. It was concluded that the liver at this stage of development is already capable of synthesizing plasma proteins exclusive of gamma globulin.

2 Similar studies were conducted using human fetal heart for purposes of comparison. The differences were striking and conform with the picture of an organ synthesizing proteins for its own use.

3 Experiments with human placenta from pregnancies of 3 to 4 months duration have demonstrated that the placenta will incorporate glycine 2 C_{14} into proteins with alpha and beta globulin mobilities. These proteins are immunologically unrelated to plasma proteins.

4 Similar experiments with term placentas revealed incorporation of radioactivity into proteins with similar electrophoretic mobility. However these proteins are related immunologically to plasma globulins.

5 At neither stage of pregnancy does the placenta synthesize proteins that are electrophoretically identifiable as albumin or gamma globulin.

6 It is concluded that under normal circumstances the placenta does not contribute significantly to the plasma proteins of the fetus. Beginning early in gestation the fetal liver is capable of synthesizing all plasma proteins except gamma globulin. The gamma globulin is probably derived from the mother.

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ANTIBODY PROTEIN SYNTHESIS BY LYMPH NODES HOMOTRANSPLANTED TO A HYPOGAMMAGLOBULINEMIC ADULT

By CHRISTOPHER M. MARTIN JOHN B WAITE AND NORMAN B McCULLOUGH

(From the Department of Health Education and Welfare Public Health Service National Institutes of Health National Institute of Allergy and Infectious Diseases and National Cancer Institute Bethesda Md)

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After showing that normal skin homografted upon a congenitally agammaglobulinemic child survived for a prolonged period (1) Good and Varco further demonstrated that within certain limits hypogammaglobulinemic patients might be artificially endowed with a system of active immunity through homotransplantation of a reticulo-endothelial tissue (2 3)

The present study was designed both to explore the therapeutic possibilities of lymph node homotransplantation and to take advantage of the unique antibody synthesizing defect in hypogammaglobulinemia to quantitate the immune response of human lymphoid tissue

This paper presents the results of measurements of antibody protein synthesis during primary and secondary immune responses by normal lymph nodes homotransplanted to a hypogammaglobulinemic adult together with qualitative observations of the interplay of recipient and transplant during and after the 150- to 160-day period of survival of the lymph nodes

MATERIALS AND METHODS

Challenge antigen Because it is potent, reliable, and rapidly productive of antibodies susceptible to accurate measurement, typhoid vaccine was selected as the antigen with which to test the reactivity of the transplant. The preparation employed[1] to challenge both recipient and donor contained 1 000 million heat killed organisms of the Panama 58 strain per ml., and was of the type ordinarily stimulating production of H and O (but not V_1) antibodies.

Titration of typhoid H and O-agglutinins. Agglutinins were measured on coded specimens by one observer at one time, and expressed as the mean of four determinations on recipient sera and two on donor sera, by the slide agglutination method devised by Welch and Stuart (4) and improved by Diamond (5) Single pooled batches of commercial (Lederle) Salmonella Group D somatic antigen and of typhoid H antigen were employed. The test was further refined as follows the titration was begun at 1 10 employing 0 16 ml of serum and all subsequent successively smaller serum aliquots were made up to 0 16 ml. with saline prior to the addition of 0 03 ml of antigen moreover dilutions of 1 15 1 30 1 60 etc. employing appropriate serum aliquots were interpolated between the conventional 1 10 1 20 1 40 etc. dilutions Under these more rigorous conditions end points (50 per cent agglutination) were sharp and repeated titrations reasonably reproducible.[2] The readings obtained were deemed internally consistent though not necessarily quantitatively identical with values ordinarily obtained with different batches of tube or slide antigens

No satisfactory method of accurate titration of low (< 1 10) titers of H agglutinins was found. Titers of O agglutinins as low as 1 2.5 however could be measured satisfactorily by prior 2 or 4 fold concentration of the serum beta-globulins through the use of zinc proteinate reactions based on Cohn plasma fractionation Method 12 (6) as follows

A portion of cold buffered zinc diglycinate-zinc acetate solution, containing 500 mM Zn per L. was added to the serum at 0 to 2 C to a final Zn concentration of 20 mM per L. The mixture was allowed to equilibrate 30 minutes and was centrifuged at 0 to 2 C for 15 minutes at 1 400 *g* The supernatant was discarded. The precipitate was made up to one-fourth or one half the original serum volume with 0.3 M sodium citrate at pH 7.2. Recovery of both H and O agglutinins appeared to be complete, but the concentrate was suitable for slide agglutination titration of only O antibody The presence of citrate ion apparently inhibited the reaction of H-antigen and antibody the citrate effect could be reversed by dialysis, but only at the expense of reduced concentration

Titration of tissue antibody Tissue extracts suitable for titration of H and O-agglutinins were prepared as fat free saline suspensions by the method of Mountain (7)

Immunochemical estimation of typhoid O-antibody To define a ratio between units of serum typhoid O agglutinin activity (reciprocal of titer) and micrograms of typhoid O $beta_2$ globulin nitrogen per ml., the method of analysis of specific precipitates (8 9) was adapted as follows

The antigen used consisted of a saline suspension of the colonies scraped from a 20-hour agar slant culture

[1] Typhoid Vaccine, Eli Lilly and Co., Lot No. 7289-62783.

[2] See Table III

of *Salmonella typhi*, H901W strain. The suspension was heated two and one half hours at 100° C to destroy traces of flagellar antigen was centrifuged and was twice washed with saline. Saline was then added to yield a suspension containing approximately 60 μg N per ml

Preliminary experiments under conditions of antibody excess and antigen excess indicated a wide equivalence zone, as reported by Landy Johnson, Webster, and Sagin (10), with prozone and postzone phenomena occurring only under extreme conditions in addition, the very low antibody N antigen N ratio in equivalence-point precipitates noted by Gurevitch and Ephrati (11) was confirmed.

Sera taken from donor and recipient were used in 0.2- to 5 0 ml amounts Sera were inactivated 30 minutes at 37° C and prior to antigen addition were centrifuged free of traces of particulate debris Mixtures of serum, saline and 1 0 ml antigen were incubated 1 hour with occasional agitation, in a 37° C bath, and then for 18 hours at 4° C

The mixtures were spun 30 minutes at 3° C and 1 400 *g* The supernatants were decanted and checked for residual agglutinating activity and the precipitates were washed twice with cold saline and transferred quantitatively to Kjeldahl digestion flasks

Nitrogen was determined by a micro-Kjeldahl procedure employing powdered selenium as a catalyst. The color reaction with Nessler s reagent was developed in the cold and read immediately at 500 mμ in a spectrophotometer

Immunochemical estimation of typhoid H-antibody The method described above was used with the following differences

Antigen was prepared by adding an equal volume of 0 6 per cent formalinized saline to a 20 hour trypticase-soy broth culture of the H901W strain of *S typhi* the motility of which had been increased to a maximum by repeated passage in semi solid agar The organisms were

FIG. 1 SITES OF TRANSPLANTATION OF LYMPH NODE SLICES IN MEDIAL THIGHS

centrifuged and washed free of broth protein, and saline was added to yield a suspension containing approximately 50 μg N per ml The sera measured were those noted above from which O antibody had been completely absorbed

Tuberculin testing Tuberculin tests of the recipient were performed with 0 1-ml intradermal doses of fresh solutions of a single lot of Sharp and Dohme Second-Strength Tablets Tuberculin Purified Protein Derivative (0 005 mg per 0 1 ml), and 0 1 ml doses of fresh saline dilutions of that lot equivalent in potency to P P D Intermediate (0 0001 mg per 0 1 ml) and P P D No 1 (0 00002 mg per 0 1 ml) Induration was measured by the method of Lovell, Goodman, Hudson, Armitage and Pickering (12) at 24 48, and 72 hours, and units of tuberculin reactivity roughly quantitated as millimeters mean maximum induration per log micro microgram P P D a unit based on the linear relationship between that measurement of the response and the logarithm of the dose, as described in man (12) and in animals (13) [3] Two dilutions were used for each skin test, and on each occasion the two responses agreed within 5 to 10 per cent

Histological methods Tissues for histological examination were fixed in 70–30 absolute alcohol-formalin solution and serially sectioned Alternate sections were stained with hematoxylin eosin and methyl green pyronine, and selected sections were stained with iron hematoxylin, eosin-methylene blue, and Giemsa stain Several sections were examined by polaroid and phase microscopy Attempts to count plasma cells, reticulum cells and lymphocytes were abandoned since no one stain differentiated the cell types sufficiently well to insure accuracy on successive counts

Leukocyte suspensions Fresh suspensions of viable leukocytes suitable for skin testing and for agglutinin studies were prepared from whole blood by the dextran-dextrose sequestrene technique of Brecher, Wilbur, and Cronkite (16) For skin testing the 02 ml of packed leukocytes derived from 10 ml blood were resuspended in 0 1 ml normal saline and injected intradermally The same volume of leukocytes washed and resuspended in 2 0 ml saline, served as an antigen for slide agglutinations

Statistical methods In the construction of the curve which best fits the observed antibody titers, standard methods of graphic analysis of multiple linear regression curves were employed to resolve the changing slopes

THE RECIPIENT THE DONOR AND THE CLINICAL CONDITIONS

Hypogammaglobulinemic recipient The recipient of the transplant was a 64 year-old white woman with ac-

[3] Under this schema, maximum responses of 10 × 10 mm induration to 0 1-ml doses of P P D No 2, Intermediate, and No 1 are equivalent, respectively, to 2.7 5 0 and 7 7 units of tuberculin reactivity, and the 1 +, 2 +, 3 +, 4 + criteria employed by Lawrence in passive transfer experiments (14 15) are equivalent, respectively, to 1 4-2 7, 2.7–5 4, 5 4–8 1, and > 8 1 units

quired hypogammaglobulinemia,[4] whose case history has been reported elsewhere (17) In addition to her basic disease, the following secondary complications and unrelated conditions were present a stable mediastinal mass (presumably a hyperplastic now fibrotic thymus) chronic pyelonephritis (with normal blood urea nitrogen and only mild impairment of renal function) bronchiectasis hypersplenism (with a moderate hemolytic anemia and a cyclic neutropenia) and arteriosclerotic heart disease, with mild ankle edema despite therapeutic doses of digitoxin.

Throughout the 137-day period preceding the transplantation and the 231-day period following it, the recipient received sulfadiazine sufficient to maintain a near-constant serum sulfonamide level of 5 to 8 mg per 100 ml and human gamma globulin[5] 5 0 gm. intramuscularly every 14 days, a dose which maintained a constant serum gamma globulin level of 0.30 ± 0.02 gm. per 100 ml. (17) and a constant exogenous typhoid H-agglutinin titer of 1 10 She had previously proved totally unresponsive to primary and repeated booster doses of typhoid paratyphoid vaccine and to several 0 1 ml. intradermal doses of P P.D No 2

There began to develop 30 to 40 days prior to transplantation, a severe neutropenia which, instead of remitting as in the past, persisted until the 257th day after transplantation

Choice of donor The following major and minor criteria were formulated for the selection of a donor for safe and successful transplantation

Major 1) No evidence of active tuberculosis, of other acute or chronic transmissible infection or of neoplastic disease 2) Neither evidence of past or of present hepatitis nor history of transfusions within the past 6 months 3) Close genetic relationship to the recipient 4) Requirement of abdominal surgery for other benign reasons, to which lymph node excision would be incidental.

Minor 1) Identical blood type (B Rh positive) 2) Positive tuberculin reaction (or other delayed type cutaneous hypersensitivity) 3) Age between 20 and 50 years 4) Absence of typhoid agglutinins and no history of typhoid infection or immunization.

The donor selected was the patient s 62 year-old sister who had always been in good health and who required an elective hysterectomy and perineal repair for a third degree uterine prolapse and cystocoele. Studies revealed Type A Rh positive blood, normal liver function a positive reaction to P P D No 1 and X ray evidence of healed fibrotic disease at the apex of the right lung There was no history of typhoid fever or of typhoid immunization, and typhoid H and O agglutinins were absent in 1 10 and 1 2.5 dilution, respectively Titers of somatic agglutinins against other Salmonellae were A <1 5 B <1 5 C (C_1 C_2) 1 40 and E (E_1 E_2, E_3) <1 5

Except in age and blood type, the donor therefore satisfied all the major and minor criteria.

Homotransplantation procedure On June 22 1955 a left supraclavicular lymph node was excised from the recipient. One-half was fixed promptly in alcohol formalin and the other frozen for later antibody studies

Simultaneously in an adjacent operating room, the surgeon[6] excised a portion of the fat pad containing the donor's left hypogastric lymph node chain and immersed the specimen in Ringer's solution. A total of 16 symmetrically placed, 2.0 to 2.5-cm. subcutaneous incisions had meanwhile been made in the inner aspect of the recipient s thighs (Figure 1) and packed with cotton gauze sponges soaked in Ringer s solution To minimize tissue trauma, hemostats and ligatures had been used sparingly

Each of nine small lymph nodes was dissected free of the fat pad cleaned of traces of pericapsular fat, rapidly and sterilely weighed on a Roller Smith automatic precision balance, and placed separately in individual screw cap vials each vial containing 1.5 ml of the recipient s serum to which penicillin and dihydrostreptomycin had been added to a concentration of 100 μg per ml

The wet weights of the nodes (in mg) were respectively 36.9 41.8 64 1 110 1 120.2 127.8 130.1 213.8, and 82.5 The 82.5-mg node was divided in halves and grossly examined. One half was placed in alcohol formalin and the other frozen and saved.

Working rapidly the operator transplanted each node in turn as follows with a minimum of trauma each node was sliced into four strips each strip no greater than 2 mm. thick Each strip was briefly checked for gross pathology and then two strips were placed in an incision in the left thigh and two in the symmetrical incision in the right No further chemotherapy was given the recipient and no wound infections occurred. Healing progressed uneventfully and at no time did sloughing occur

Antigenic challenge of recipient and of donor Seven days after transplantation, the recipient was challenged with 0.5 ml of the previously described typhoid vaccine injected subcutaneously in the left arm. A booster dose of 0.5 ml was given seven days later and another twenty days later

To avoid problems of interpretation of titers in the donor however initial 0.5 ml challenge was delayed until the ninth day when the postoperative phase of heightened adrenal cortical activity had presumably passed. Booster doses of 0.5 ml. were given seven and, through an oversight, eighteen (rather than twenty) days later

Excision biopsies Nineteen days after transplantation, the strips from the node originally weighing 110 1 mg were excised from their two symmetrical sites. At the same time a right supraclavicular node was excised from

[4] Patient referred for study by Stuart O Foster M D., Washington D C.

[5] Poliomyelitis Immune Globulin, Squibb and Sons Lot No 252-2 kindly supplied by the American National Red Cross.

[6] J Keith Cromer Department of Gynecology and Obstetrics, George Washington University School of Medicine, whose cooperation the authors gratefully acknowledge.

the recipient. Half of each of the specimens was fixed in alcohol formalin the remainder was subdivided grossly into node fragments fat and skin and was frozen and saved.

RESULTS

Immune responses

Tuberculin tests of the recipient two days after transplantation disclosed a powerful passively transferred delayed cutaneous hypersensitivity to tuberculin (Figure 2) Tuberculin reactivity then steadily increased to a plateau which extended from the 68th day to the 149th at a level equivalent to a reaction of 25 × 25 mm induration to P P D No 1 Between the 149th and the 217th days reactivity fell off to a plateau at a lower, though still highly reactive, level Reactivity was essentially unaffected by splenectomy on the 257th day

In response to early challenges with typhoid vaccine administered at sites distant from the transplants, the recipient developed low titers of typhoid H- and O-agglutinins (Figure 2) in a manner qualitatively similar to the higher titers observed in the donor (Figure 3) A booster challenge on the 98th day again elicited a secondary response, after which titers began to decline more rapidly A final challenge on the 168th day elicited no response, instead, measurable H-agglutinins declined exponentially over the next 80 days with a slope statistically indistinguishable from that of passively infused H-agglutinins in other hypogammaglobulinemic patients (described below)

Biopsies

The donor's ninth hypogastric lymph node, which had not been transplanted, was histologically normal and quiescent and contained occasional plasma cells, a saline extract of the node was devoid of typhoid H- and O-antibody (< 1 20)

The recipient's left and right supraclavicular nodes excised before and 19 days after transplantation, were virtually identical, both contained a total of only one or two dubious plasma cells and no antibody (< 1 20) Both contained abundant

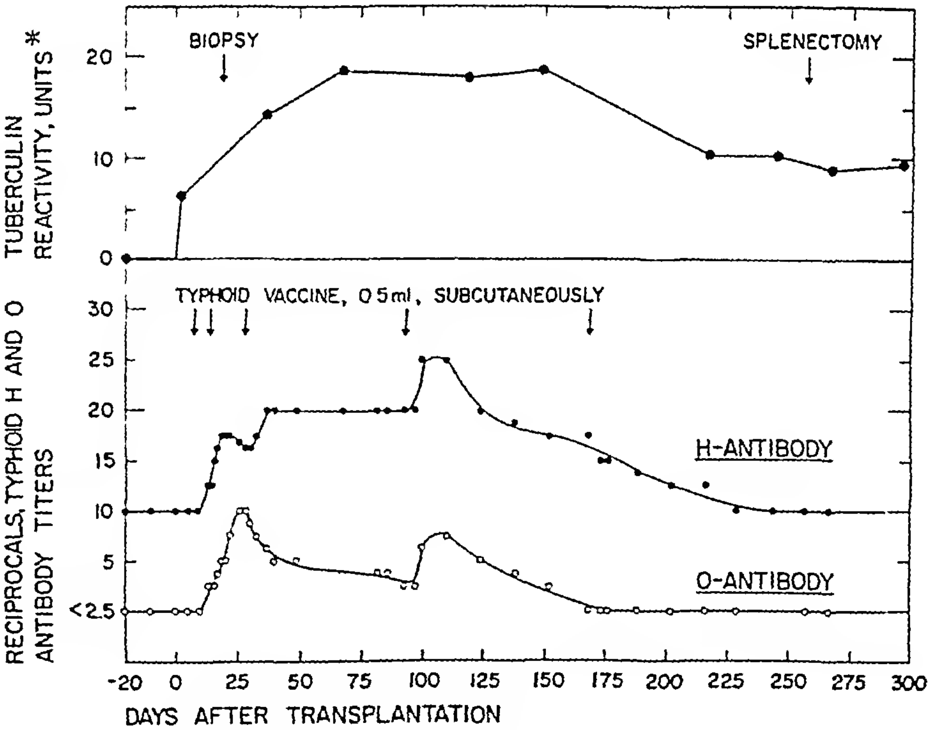

FIG ? IMMUNE REACTIONS OF TRANSPLANTED LYMPH NODES

*Tuberculin r… s V… induration per log micro-microgram P P D Each (… o dilutions

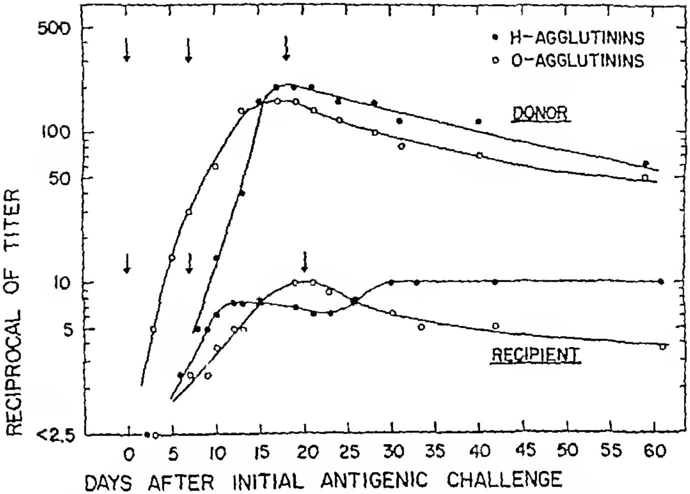

FIG. 3 DONOR AND RECIPIENT ANTIBODY TITERS AFTER TYPHOID IMMUNIZATION
The H agglutinin titers observed in the recipient (Figure 2) are here corrected for a constant exogenous titer of 1 10

lymphocytes and reticulum cells but sparse and poorly developed germinal centers

The 19th day excision biopsy of one of the transplanted nodes disclosed evidence of an initial take the tissue contained moderate numbers of plasma cells and abundant tissue antibody Extracts of various fragments grossly identified as node tissue (the bulk of each of which however was granulation tissue) titered 1 40 1 40 1 80 and 1 80 against H antigen and 1 20 1 40 1 80 and 1 200 against O antigen Extracts of adjacent specimens of fat and of the overlying skin and scar contained no demonstrable antibody Serum H and O titers at the time of biopsy were 1 17 5 and 1 5 respectively

Microscopically the transplant (Figure 4) contained architecturally disorganized cords of lymphoid tissue intimately penetrated by granulation tissue, with occasional foreign body giant cells at the periphery Higher magnification (Figure 5) disclosed intensely vascularized cords and strands of reticulum cells lymphocytes and scattered (but not abundant) plasma cells. Study of the giant cells by polarized light suggested that they were not reacting specifically to the donor tissue each contained a bit of refractile cotton fiber, presumably from the surgical sponges used at the time of transplantation

Therapeutic effects of transplantation

Despite a severe neutropenia (white blood counts 1200 to 2400 with 2 to 18 per cent neutrophils) the recipient remained free of major infections throughout the life of the transplant During that interval there occurred episodes of oral moniliasis from the 76th day to the 81st and from the 130th to the 134th a syndrome of fever pharyngitis cervical lymphadenopathy and atypical lymphocytosis from the 86th day to the 96th, and facial furuncles from the 106th day to the 110th.

In contrast, the 100-day period following the presumed death of the transplant was filled with a succession of increasingly severe infections—ulcerative pharyngitis and stomatitis recurrent oral moniliasis facial furuncles severe acute pansinusitis due to *Staphylococcus aureus* and mul

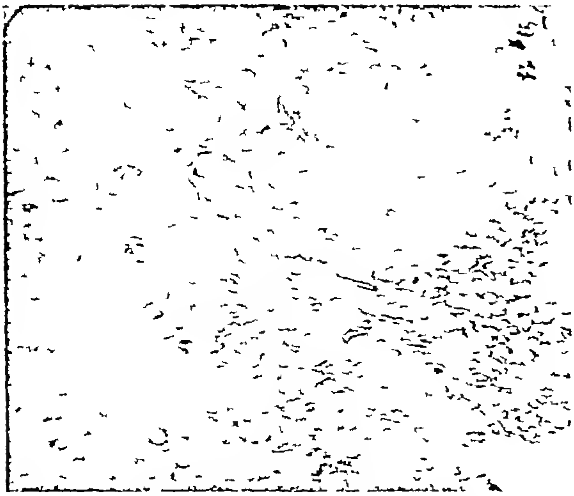

FIG 4 EXCISION BIOPSY, TRANSPLANTED LYMPH NODE SLICE, NINETEENTH DAY

From left to right are seen granulation tissue, scattered giant cells, cords of lymphoid tissue and partially necrotic fat (H & E × 85)

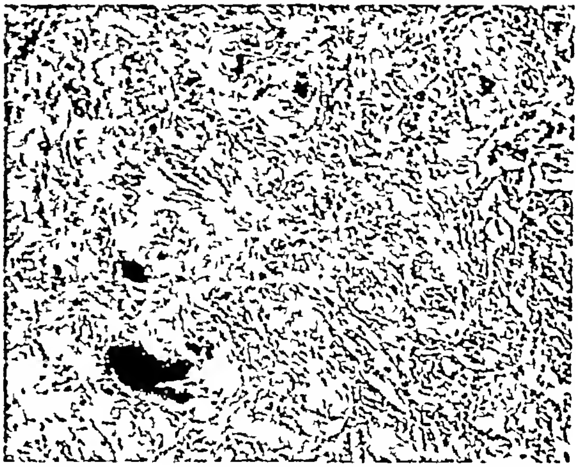

FIG 5 EXCISION BIOPSY, TRANSPLANTED LYMPH NODE SLICE, NINETEENTH DAY

Within the intensely vascularized, disorganized cords of lymphoid tissue are reticulum cells, plasma cells and foreign-body giant cells (H & E × 150)

tiple subcutaneous staphylococcal abscesses—which together with the recipient's first severe thrombocytopenic episode culminated in splenectomy on the 257th day. Postoperatively there occurred a prompt, sustained hematologic and symptomatic remission

Also in contrast, during a comparable, non-neutropenic 96-day period before transplantation there occurred only a single mild upper respiratory infection lasting five days (17)

Neither during the life of the transplant nor after splenectomy did there occur any detectable rise in the recipient's serum gamma-globulin level

In an attempt to make therapeutic use of the transplant, the patient was immunized, at bi-weekly intervals beginning on the 68th day, with standard parenteral doses of the following antigens: diphtheria toxoid-tetanus toxoid-pertussis vaccine, polyvalent influenza vaccine, and two doses of a combined respiratory bacterial vaccine[8]. In addition, four doses of an autogenous, formalinized *Escherichia coli* (communis) vaccine, prepared from the strain isolated from the recipient during a pyelonephritis and bacteremia fourteen months previously (17) and still present in the urine, were given during this period

Although there was suggestive evidence of weak responses to several of the administered antigens, extensive titrations of pre- and post-challenge sera failed to demonstrate unequivocal antibody formation

Immune interactions of transplant and recipient

To explore the possibility that the transplant might be rejecting the recipient, as well as *vice versa,* attempts were made to demonstrate isoimmune phenomena in several systems. Neither clinical nor laboratory evidence of nephritis, encephalitis, or collagen diseases appeared, and no evidence was adduced to implicate the transplanted nodes in the persistence of the recipient's neutropenia. The results of repeated Coombs tests were negative, and the recipient's first episode of thrombocytopenia occurred three months after transplant function had ceased

Multiple histological sections of the 630 gm spleen revealed increased numbers of Malpighian follicles, prominent germinal centers, normal numbers of reticulum cells and lymphocytes, and prominent erythrocytophagocytosis by monocytes. Only a very few cells resembling plasma cells were seen, and there was no evidence of lymphoma, granuloma formation, amyloidosis or collagen disease. A specimen of the liver was histologically normal. Cultures and animal inoculations of organ suspensions were negative for bacteria, mycobacteria, fungi and *Toxoplasma*

[8] Immunovac Respiratory Vaccine®, Parke Davis & Co

Despite the challenge of small numbers of incompatible (Type A) red cells the recipient failed to develop anti A isoagglutinins in 1 1 dilution measured by both the saline and the indirect Coombs techniques

By the use of viable donor leukocytes as a slide agglutination antigen it was demonstrated that the recipient failed to develop circulating leukocyte agglutinins in 1 1 dilution at any time after transplantation

Skin tests however 250 and 266 days after transplantation, demonstrated a reproducible specific delayed tuberculin-like cutaneous hypersensitivity to viable donor leukocytes Intradermal injection of the leukocytes derived from 10 ml of fresh donor blood elicited a reaction beginning at three hours and reaching a maximum diameter at 24 to 36 hours of 14 to 16 mm of erythema and 10 to 12 mm. of tender induration, with a central hemorrhagic spot. Only transient 3 to 4 mm diameter erythematous responses were elicited by control injections of approximately equal numbers of leukocytes derived from the recipient and from normal Type O tuberculin positive Type B tuberculin negative and Type A tuberculin negative persons as well as of donor plasma containing dextrose dextran sequestrene *

Immunochemical studies

The results of immunochemical studies (Figures 6 7) indicate that under the conditions of biological assay described above 1 unit of typhoid H agglutinating activity synthesized by the specific donor (or donor tissue) is equivalent to 126 ± 009 microgram gamma globulin N per ml serum and that 1 unit of similarly specific typhoid O agglutinating activity is equivalent to 102 ± 026 microgram beta_2 globulin N per ml serum With the whole bacilli antigens employed the ratios in equivalence-point precipitates of antibody N antigen N for the H and O systems were respectively 1 2.85 and 1 5 27

Estimation of rates of antibody synthesis

In the absence of demonstrable recipient antibody synthesis and with the knowledge that extracts only of the transplants contained large amounts of antibody it was concluded that all observed titers represented antibody synthesized by the transplant A general equation for calculating daily total synthesis of typhoid antibody by the transplant was therefore formulated as follows

If S be the weight of H or O antibody synthesized and released per gram of lymph node tissue per day I the daily increment in serum titer in agglutinating units D the units of antibody passively degraded per day V the apparent body fluid volume or compartment in which the antibody is diluted K the constant that relates units of agglutinating activity to weight of antibody nitrogen 6.2 the protein nitrogen ratio in both gamma and beta_2 globulin (18) and W, the weight in grams of transplanted lymph node tissue then on any given day

$$S = \frac{(I + D)(V)(K)(6.2)}{W} \qquad (1)$$

Values of I were read from the curve of antibody titer derived from graphic analysis of the observed titers (Figure 3)

Values of D were calculated by substituting each day's average serum titer for C in the equation describing one day's exponential decline of passively acquired antibody from a level of C to a level of C_0

$$\frac{\text{Log } C - \text{Log } C_0}{1} = \text{Slope} = \text{Constant} \qquad (2)$$

solving for C_0 and subtracting from C The appropriate constant for H antibody was determined and that for O antibody was assumed as follows

H antibody After administration of 50 gm of human gamma globulin * to two other hypogammaglobulinemic patients the passive rates of decline of serum gamma globulin and of six common antibodies were determined by *in vitro* assays of multiple periodic serum specimens drawn over a 63 to 70-day period (19) The half life of typhoid H agglutinin so determined was 35 0 ± 3 6 days corresponding to a slope of 0 0086

O-antibody Although the half life of beta_2 globulin in human beings has never been determined sparse indirect evidence suggests that beta_2 globulins are passively degraded considerably faster than gamma globulins The equations with

* Because of the possibility of immunizing the recipient against her prospective donor the recipient was not skin tested with donor leukocytes before transplantation.

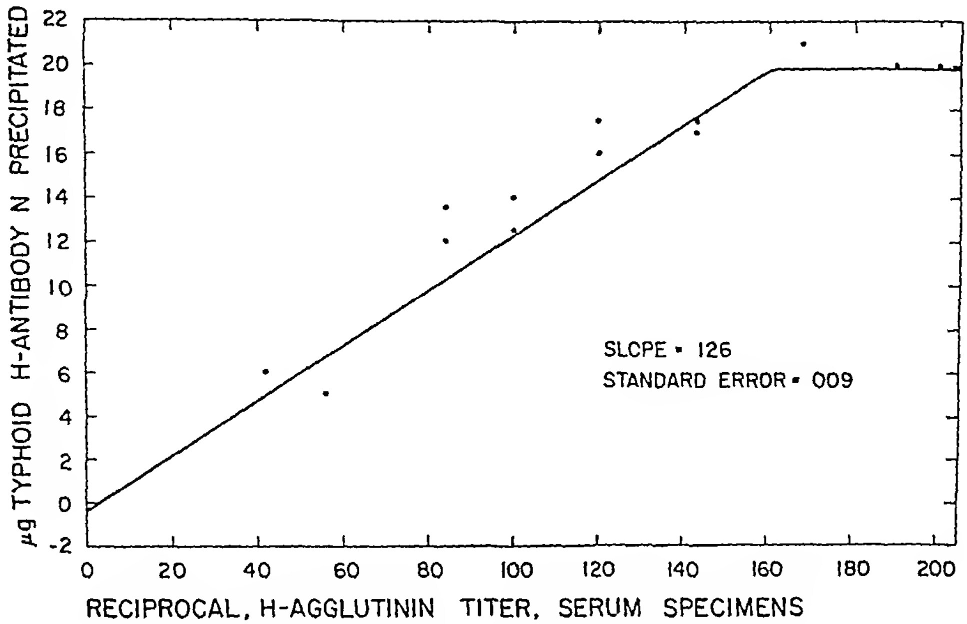

FIG 6 ANTIBODY NITROGEN PRECIPITATED WITH 56 5 MICROGRAMS TYPHOID H-ANTIGEN NITROGEN

which Wiener demonstrated that newborns passively degrade diphtheria antitoxin at a rate identical with that of adults (20), when applied to the data of Smith on the decline of passively acquired compatible isoagglutinins in newborns (21), indicate that these beta$_2$-globulins have a half-life of 12 to 18 days Biosynthetic determinations of the half-lives of heterogeneous plasma protein fractions, of which beta$_2$-globulins were only one of many components, have yielded values of 6 2 to 7 8 days for fractions I + III (22) prepared by Cohn Method 10 (23), and 17 days for pooled alpha$_1$-alpha$_2$-beta-globulins (24)

After two transfusions of fresh Type B, Rh positive blood, the recipient's passively acquired anti-A isoagglutinin titers were followed for 28

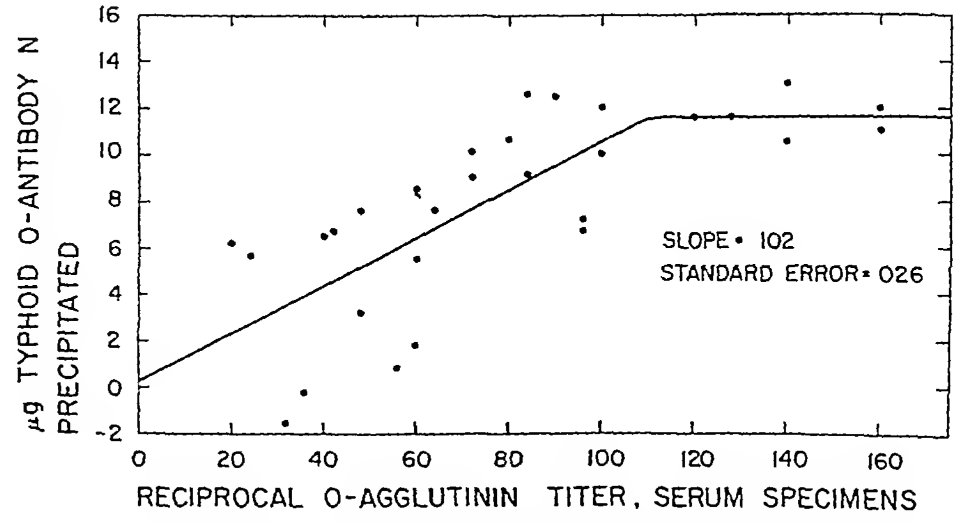

FIG 7 ANTIBODY NITROGEN PRECIPITATED WITH 61 1 MICROGRAMS TYPHOID O-ANTIGEN NITROGEN

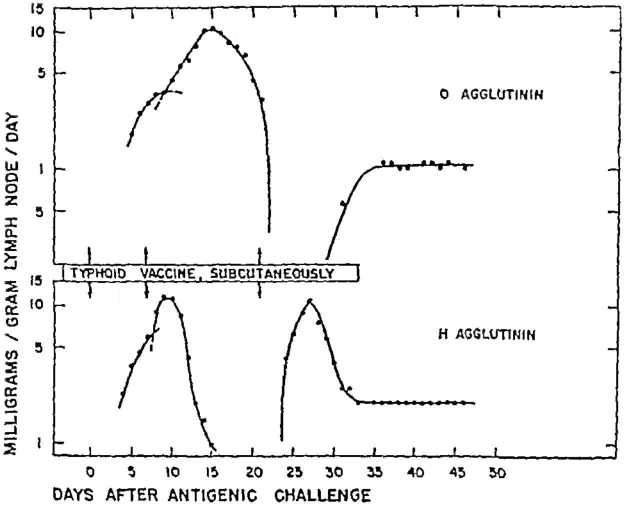

Fig. 8. Antibody Protein Synthesis by Transplanted Lymph Nodes

days Analysis of seven determinations yielded a linear regression curve with a half life of 16 7 days and a standard error of 2 5 days

Employing these data and noting that the maximum slopes in the declining phase of the curves of O-antibody titer in both donor and recipient corresponded to half lives of 15 ± 2 days (Figures 2 and 3) a 15-day half life of O agglutinins (or slope of 0 020) was assumed

Compartment size Previous studies of the recipient had demonstrated that after equilibration administered gamma globulin (including typhoid H antibody) behaves as if diluted in a fluid volume equivalent to 20 per cent of body weight (17), *i e*, 25 to 30 per cent is found in the plasma and the remainder in extravascular compartments In this study in which the recipient s weight (47 Kg) remained essentially constant throughout the period of observation the volume of distribution V of typhoid H antibody was therefore equivalent to 9 4 liters

It was not possible to determine directly V for typhoid O antibody since no safe concentrated source of human O antibody is available for human administration As a near approximation, the V for anti A isoagglutinins, which are chemically and electrophoretically similar to typhoid O-agglutinins was determined After transfusion of the recipient with fresh blood containing compatible plasma of known isoagglutinin content anti A isoagglutinins behaved as if diluted in a volume equivalent to 20 ± 3 per cent of body weight (25) Since this figure is compatible with the known presence of typhoid O antibody and isoagglutinins in numerous tissues and interstitial fluids (26–29), it appeared reasonable to assume V for typhoid O-antibody to be identical with that of H antibody

Employing these data and assuming further prompt (24-hour) mixing of newly synthesized antibody and full survival of the transplant (W = 844.8 mg before excision biopsy and 734.7 after) minimum rates of antibody synthesis were calculated (Table 1) and graphed (Figure 8)

DISCUSSION

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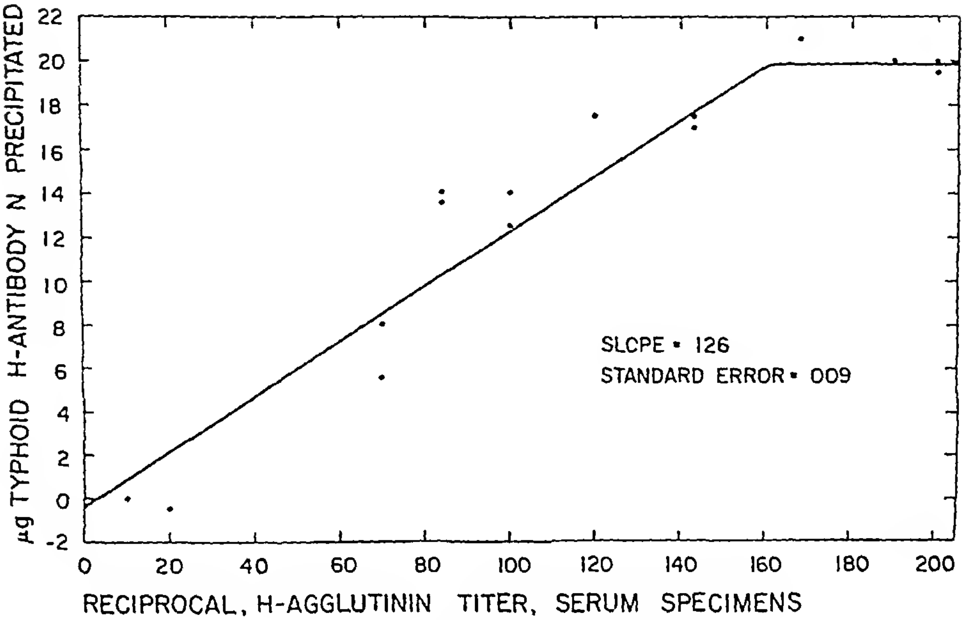
The present study confirms the demonstration by Good and Varco (1–3) of the impaired ability of the hypogammaglobulinemic patient to respond



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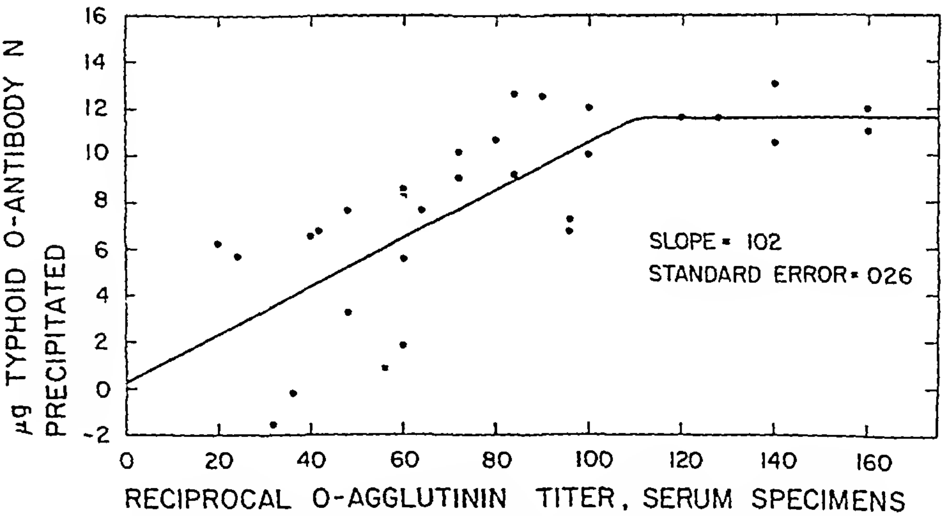

Fig 7 Antibody Nitrogen Precipitated with 61 1 Micrograms Typhoid O-Antigen Nitrogen

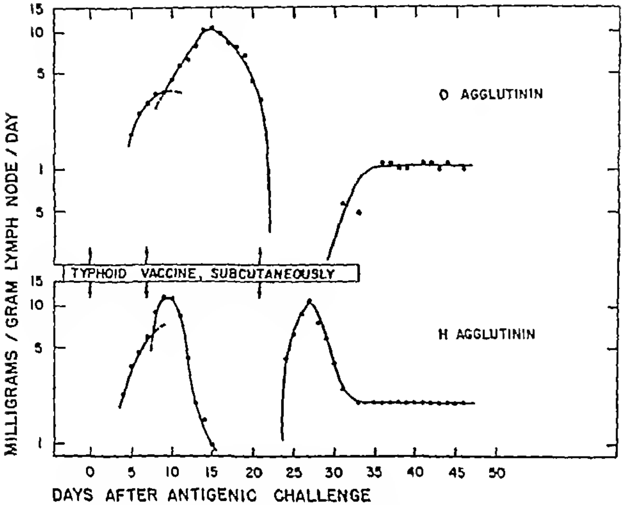

FIG. 8. ANTIBODY PROTEIN SYNTHESIS BY TRANSPLANTED LYMPH NODES

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DISCUSSION

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TABLE I

Serum titrations and estimated daily rates of typhoid H- and O antibody synthesis by transplanted lymph nodes

Days after initial antigenic challenge	Reciprocals of four serum titrations (*units/ml*)		Reciprocal mean observed endogenous serum titer (*units/ml*)		Reciprocal estimated graphic titer (*units/ml*)		Antibody destroyed per day (*units/ml*)		Total antibody synthesized per day (*units/ml*)		Milligrams antibody protein synthesized/gram wet weight lymph node/day	
	H	O	H	O	H	O	H	O	H	O	H	O
2	10, 10, 10, 10		0									
3	10, 10, 10, 10	< 2 5 × 4	0	<2 5	1 0							
4					1 25	1 25	02		27		2 32	
5		< 2 5 × 4		<2 5	1 65	1 45	03	06	43	26	3 70	1 79
6	10, 10, 15, 15		2 5		2 15	1 75	04	07	54	37	4 64	2 55
7	10, 10, 15, 15	2 5, 2 5, 2 5, 2 5	2 5	2 5	2 80	2 10	05	09	70	44	6 02	3 03
8					3 80	2 50	06	11	1 06	51	9 11	3 52
9	15, 15, 15, 15	2 5, 2 5, 2 5, 2 5	5 0	2 5	5 05	2 90	09	12	1 34	52	11 52	3 59
10	15, 15, 15, 20	2 5, 3 75, 3 75, 5	6 25	3 75	6 25	3 40	11	14	1 31	64	11 27	4 42
11					7 10	4 05	13	17	98	82	8 43	5 65
12	15, 15, 20, 20	5, 5, 5, 5	7 5	5 0	7 45	4 75	14	20	49	90	4 21	6 20
Biopsy												
13	15, 15, 20, 20	5, 5, 5, 5	7 5	5 0	7 50	5 50	15	23	20	98	1 98	7 77
14					7 50	6 50	15	27	15	1 27	1 48	10 07
15	15, 15, 20, 20	5, 7 5, 7 5, 10	7 5	7 5	7 45	7 50	15	32	10	1 32	99	10 46
16					7 25	8 35	14	36	− 06	1 21	− 59	9 60
17					7 10	9 00	14	39	− 01	1 04	− 10	8 25
18					6 95	9 55	14	42	− 01	97	− 10	7 70
19	7 5, 20, 20, 20	10, 10, 10, 10	6 88	10	6 75	9 95	13	44	− 07	84	− 69	6 66
20					6 60	10 05	13	45	− 02	55	− 20	4 36
21	15, 15, 15, 20	10, 10, 10, 10	6 25	10	6 40	10 00	13	45	− 07	40	− 69	3 17
22	15, 15, 15, 20		6 25		6 25	9 35	12	44	− 03	− 21	− 30	−1 67
23		7 5, 7 5, 10, 10		8 75	6 20	8 75	12	41	07	− 19	69	−1 51
24					6 50	8 30	12	39	42	06	4 16	48
25					7 00	7 80	13	36	63	− 14	6 24	−1 11
26	15, 15, 20, 20	7 5 × 4	7 5	7 5	7 75	7 40	14	34	89	− 06	8 82	− 48
27					8 70	7 05	16	32	1 11	− 03	10 98	− 24
28					9 30	6 70	18	31	78	− 04	7 72	− 32
29					9 70	6 40	19	30	59	0	5 84	0
30	20, 20, 20, 20	5, 5, 7 5, 7 5	10	6 25	9 90	6 15	19	28	39	03	3 86	24

TABLE 1—*Continued*

Days after initial antigenic challenge	Reciprocals of four serum titrations (units/ml.) H	Reciprocals of four serum titrations (units/ml.) O	Reciprocal mean observed endogenous serum titer (units/ml.) H	O	Reciprocal estimated graphic titer (units/ml.) H	O	Antibody destroyed per day (units/ml.) H	O	Total antibody synthesized per day (units/ml.) H	O	Milligrams antibody protein synthesized/gram wet weight lymph node/day H	O
31					9 95	5 95	20	.27	25	07	2.48	.56
32					10 0	5 80	.20	.27	.25	12	2 48	95
33	20 20 20 20	5 5 5 5	10	5 0	10 0	5 60	.20	26	20	06	1 98	48
34					10 0	5 50	.20	25	.20	15	1 98	1 19
35					10 0	5.35	20	24	.20	09	1 98	71
36					10 0	5.25	.20	.24	20	14	1 98	1 11
37					10 0	5 15	.20	24	20	14	1 98	1 11
38					10 0	5 05	.20	23	.20	13	1 98	1 03
39					10 0	4 95	.20	.23	.20	13	1 98	1 03
40					10 0	4 85	.20	22	20	12	1 98	95
41					10 0	4 77	20	.22	.20	14	1 98	1 11
42	20 20 20 20	5 5 5 5	10	5 0	10 0	4 70	.20	.21	20	14	1 98	1 11

Additional serum titrations

Days after initial antig. challenge		61	73	79	86	90	93	103	117	131	145	161	166	169	181	195	209	222	237
Reciprocals, *four serum* titrations (*units/ml*)	H	10 20 20 30	20 20 20 20	20 20 20 20	20 20 20 20	20 20 20 20	20 20 30 30	20 20 30 30	20 20 20 20	15 20 20 20	15 15 20 20	15 15 20 20	15 15 15 15	15 15 15 15	10 15 15 15	10 10 15 15	10 10 15 15	10 10 10 10	10 10 10 10
	O	3 75 3 75 3 75 3 75	3 75 3 75 3 75 3 75	2.5 3 75 3 75 5	2.5 2.5 2.5 2.5	2.5 2.5 2.5 2.5	7.5 7.5 7.5 7.5	5 7.5 7.5 10	5 5 5 5	3 75 3 75 3 75 3 75	2.5 2.5 2.5 2.5	<2.5 <2.5 <2.5 <2.5	<2.5 <2 5 <2.5 <2.5	<2 5 <2 5 <2.5 <2.5	<2.5 <2.5 <2.5 <2.5	<2.5 <2.5 <2.5 <2 5	<2.5 <2.5 <2.5 <2.5	<2.5 <2.5 <2.5 <2 5	<2.5 <2 5 <2.5 <2.5
Recip. mean endog. titer (*units/ml*)	H	10	10	10	10	10	15	15	10	8.75	7.5	7 5	5	5	3 75	2.5	2.5	0	0
	O	3 75	3 75	3 75	2.5	2.5	7 5	7.5	5	3 75	2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2 5	<2.5

to foreign tissue antigens and of the feasibility of artificially endowing him for a prolonged period with a functional miniature reticulo-endothelial system The data suggest that the eight transplanted lymph nodes survived with full function for 100 to 110 days, and that destruction of the tissue occurred slowly, culminating in total unreactivity about the 160th day

The recipient's passively acquired tuberculin hypersensitivity, which reached a peak only gradually, remained constant for three months, and then, coincident with cessation of node function, fell abruptly to a lower level, has continued at that level for a prolonged period In the absence of evidence for transmission of tuberculosis, this pattern of transferred reactivity conforms to one of those described by Lawrence (15) and interpreted by him and by Chase (30) as suggestive either of two distinct phases—one passive and one "active"—in the recipient's handling of a single transfer substance in intact or disrupted sensitized leukocytes or, alternatively, of two leukocyte substances—one more and one less available—involved in the transfer If it can be assumed that the transplant was totally destroyed by the 160th day, then the evidence tends to support the first hypothesis, inasmuch as only a portion of the reactivity was lost when the exogenous leukocyte source of hypersensitivity was removed

The recipient's development of a specific, delayed-type cutaneous hypersensitivity to leukocytes derived from the donor, together with her failure to develop circulating agglutinins to these leukocytes, are in accord with abundant evidence of the pre-eminent role of fixed tissue antibody rather than of circulating antibody mechanisms in the rejection of homografts and homotransplants (31–34) It was not deemed clinically justifiable to test the possibility that the recipient would manifest an accelerated rejection of another lymph node from the specific donor

All other technical considerations being equal, the limiting factor in the longevity of transplants to patients with hypogammaglobulinemia is thus apparently the degree to which this diffuse disease of the reticulum has impaired the patient's capacity to develop fixed tissue antibody Though usually markedly impaired, this capacity is well retained in a minority of patients with acquired hypogammaglobulinemia (35), moreover, its presence—though very weak—in congenitally hypogammaglobulinemic children has been demonstrated by Good (3) and Porter (36)

The histological findings in the excision biopsy neither confirm nor deny the evidence for either the plasma cell or the lymphocyte as the major cellular source of circulating antibody (37) The finding of relatively small numbers of plasma cells in microscopic sections may mean only that the pathologist's "half" of the specimen contained a small, unrepresentative portion of the transplant

Whether or not the transplant ameliorated the recipient's hypogammaglobulinemia is a moot point, her clinical course before, during and after transplantation differed in no striking way from that of progressively severe hypersplenism, or of progressive neutropenia of any etiology Conversely, it may be that the presence of neutropenia during the life of the transplant precluded effective utilization of any antibody synthesized by the transplant and that transplantation was therefore not given a fair test Whether the theoretically beneficial effects of lymph node transplantation justify the risks (principally that of contracting serum hepatitis) is thus still conjectural, at least in the case of acquired hypogammaglobulinemia In congenital hypogammaglobulinemia, on the other hand, in which one may anticipate survival of transplants for years rather than months, lymphoid tissue homotransplantation remains an attractive therapeutic possibility (35)

Laboratory observations

As predicted by Boyd and Hooker (38), who demonstrated an inverse log-log linear relationship between the ratio by weight of antibody to antigen in equivalence-point precipitates and the molecular weight of antigen, the equivalence-point ratios of typhoid H- and O-antibodies to whole bacilli antigens were very low Consequently, individual measurements of antibody precipitated from most serum specimens fell close to the limits of accuracy of the quantitative agglutinin procedure (9)—just beyond the error of the micro-Kjeldahl technique—resulting in curves the standard error of the slopes of which is relatively high 7 per cent for H-antibody and 25 per cent for O-antibody [10]

[10] Because comparably high standard deviations were associated with both repeated determinations of the antibody nitrogen in one serum and single determinations of

TABLE II

Measured rates of protein synthesis by various human and animal tissues and rates estimated from recent human biosynthetic turnover data

Tissue	Reference	Method	Protein synthesized	Experimental conditions	Rate of synthesis (*mg./gm. wet weight tissue/day*)
			Normal human tissues, *in vivo*		
Lymph node	This paper	Tissue slices homotransplanted to hypogammaglobulinemic adult	Typhoid O-beta$_2$-globulin	Immune response Peak primary response	3.6
				Peak secondary response	10.5
				Artificial acquired immunity late	1.0
			Typhoid H-gamma globulin	Peak, primary response	6.0
				Peak secondary response	11.0–11.5
				Artificial acquired immunity, late	2.0
Reticulo-endothelial system	(22)	Oral S^{35} and S^{35} labeled i.v.	Gamma-globulins	Steady state*	1.7–3.9
	(24)	Oral S^{35}			1.7–5.4
	(51)	Oral N^{15}			5.4–5.8
Liver	(22) (53)‡	Oral and i.v. S^{35} and S^{35} labeled i.v.	Total plasma protein	Steady state†	7–15
	(22)	Oral S^{35} and S^{35} labeled i.v.			3.4–6.4
	(24)	Oral S^{35}	Albumin	Steady state†	2.9–5.3
	(51)	Oral N^{15}			7–10
	(22)	Oral S^{35} and S^{35} labeled i.v.	Fibrinogen	Steady state†	1.4–2.4
			Normal animal tissues *in vitro*		
Liver (rat)	(55)	Perfused whole liver	Total plasma protein	Optimal perfusion conditions tissue from fasting rats	15–18
	(56)			Tissue from non fasting rats	50–70§
Liver (chicken)	(58)	Tissue slices	Albumin	Optimal incubation conditions	2.5–3.0
Pancreas (pigeon)	(59)	Tissue slices	Amylase	Tissue depleted of zymogen by carbamyl-choline pre-treatment	15–25
Anterior pituitary (rat)	(60)	Tissue slices	Protein hormones	Optimal incubation conditions	45–55

* Estimates assume average adult exchangeable gamma-globulin pool of 75 gm. (22) and average weight of total adult reticulo-endothelial tissue of 500 gm (52)

† Estimates assume average adult exchangeable total plasma protein pool of 530 gm albumin of 300 gm., and fibrinogen of 20 gm. (54) and average weight of adult liver of 1500 gm

‡ Serum protein

§ Steinbock and Tarver estimate rate of 45 to 65 mg per gm. wet weight liver per day in intact rat (57)

Although it is not possible to state categorically that the donor and the transplanted nodes underwent a primary immune response to typhoid vaccine, the data suggest that they did. Particularly with respect to H antibody—ordinarily the most prominent antibody developed in response to immunization—the relatively long initial induction phase in the donor and in the recipient and the

different sera of equal titer (Table IV) it was concluded that the major error in the experiment lay in the immunochemical method rather than in the agglutinin titrations.

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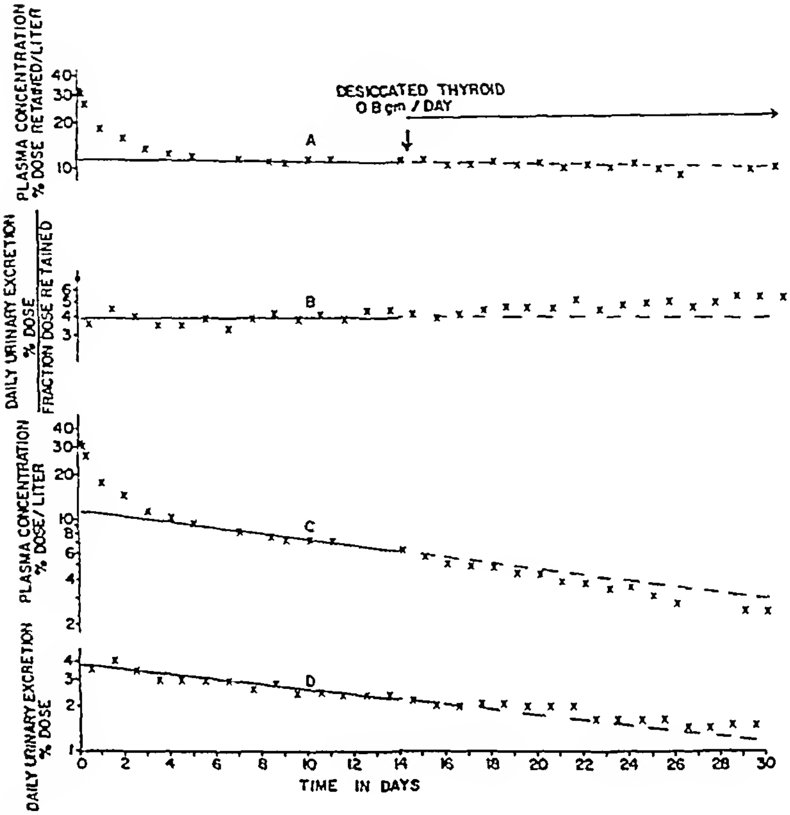

FIG 1 TYPICAL SET OF CURVES FOR PLASMA AND URINE DATA (PATIENT F M.) FOLLOWING ALBUMIN-I^{131} ADMINISTRATION (SEE TEXT)

Plasma volume and total exchangeable albumin (TEA) were determined by methods previously described (1)

The rate of metabolism of serum albumin was determined by several different means Methods employing the rate constant of decrease in plasma concentration of albumin-I^{131} after distribution equilibrium or the rate of urinary excretion of I^{131} released by degradation of albumin-I^{131} have been previously described (1) and yielded essentially identical values However, the validity of these methods depends upon the maintenance of steady state conditions During the control period, serum albumin concentrations remained constant, and for practical purposes it may be assumed that steady state requirements were satisfied. Under these conditions the rate of synthesis is equal to the rate of degradation. However, during the experimental period of thyroid administration, changes in the distribution and the rate of degradation altered the steady state so that the validity of these methods is vitiated. Therefore, the following method, the validity of which is independent of the steady state, was employed for comparison of albumin degradation during control and experimental periods Since radioactivity excreted in the urine in the absence of proteinuria represents I^{131} released by metabolic degradation of albumin-I^{131}, the amount degraded each day was calculated as the product of the apparent renal clearance of plasma I^{131} ("metabolic clearance") and the plasma concentration of albumin (Figure 2) [3] The total amount degraded over each period was then obtained from the sum of the daily values [4] The quantity of albumin synthe-

[3] It has been shown previously that the urinary excretion of I^{131} reflects very closely the degradation of albumin-I^{131} owing to the very rapid rate of renal excretion of the I^{131} released by protein degradation compared to the rate of degradation itself (1) "Metabolic clearance" methods have also been used in the study of thyroid hormone degradation (7)

[4] In subject P M the second dose of albumin-I^{131} was administered 4 days after observations on the degradation of the first dose of albumin-I^{131} were discontinued. In subject T M the total exchangeable albumin was calculated from the space of distribution of albumin-I^{131} 7 days following the administration of the second dose. In both instances the mean daily albumin degradation during the last 5 days of the treatment period was assumed to continue into these 4 and 7-day periods Although the rate of albumin degradation may have been slightly higher than the mean of the previous 5 days, this would not have introduced a significant error in the values for total albumin degraded during thyroid hormone administration.

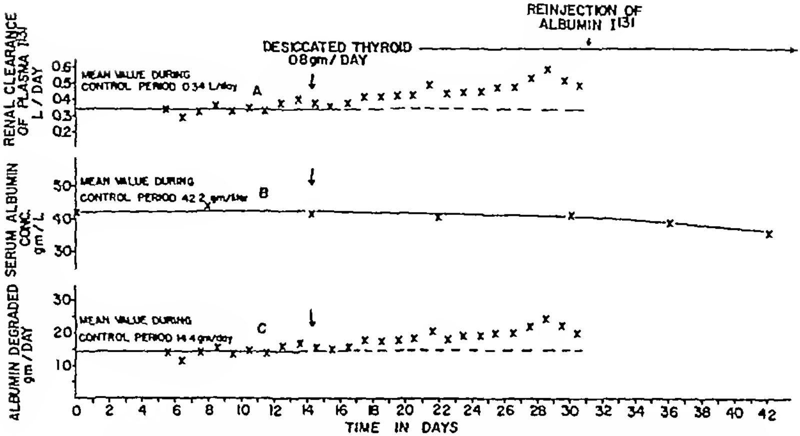

FIG. 2. PATIENT F M. DAILY URINARY CLEARANCE ("METABOLIC CLEARANCE") OF PLASMA I^{131} (CURVE A) SERUM ALBUMIN CONCENTRATION (CURVE B) AND QUANTITY OF ALBUMIN DEGRADED DAILY (CURVE C)

Following thyroid hormone therapy there was an appreciable increment in the quantity of albumin degraded even though the concentration of serum albumin decreased.

sized during thyroid administration was obtained from the difference between the total amount degraded and the change in total exchangeable albumin during this period. During the control period the amount synthesized was taken to equal the amount degraded.

RESULTS

Clinical observations and laboratory data which are not related to albumin metabolism are given in the Appendix. It is only necessary to note here that all subjects developed clinical evidence of hypermetabolism akin to that observed in hyperthyroidism within about 2 weeks following initiation of thyroid therapy

Data pertaining to albumin metabolism are summarized in Table I During thyroid administration all subjects showed a fall in total serum protein concentration the mean value decreasing from 7.03 ± 0.20 grams per 100 ml. to 6.26 ± 0.37 grams per 100 ml Serum albumin concentration fell from 4.62 ± 0.31 grams per 100 ml to 4.19 ± 0.29 grams per 100 ml. Plasma volume increased in all subjects with a mean change of + 10.4 per cent

An increase in the overall apparent space of distribution accompanied by a proportionately greater fall in serum albumin concentration resulted in a decrease in TEA of 17 gm During the control period intravascular albumin was 145 gm and extravascular albumin 205 gm After thyroid therapy the values were 143 and 189 gm. respectively indicating that the slight loss of exchangeable albumin was derived exclusively from the extravascular compartment.

During thyroid administration there was an increased metabolic degradation of albumin in all subjects This was suggested in the increased urinary excretion of I^{131} (Figure 1, Curves B D) Although the serum albumin concentration fell, the marked increase in the fractional rate of albumin I^{131} degradation more than compensated for this fall leading to an increase in the quantity of albumin degraded. Determination of the "metabolic clearance" of albumin I^{131} (Figure 2 and Table I) confirmed the increase in the absolute amount of albumin undergoing metabolic degradation. The mean value of this increase was 81 grams with a range of 32 grams to 165 grams (Table I) Since loss of total exchangeable albumin averaged 17 grams during the same period the average amount of extra albumin synthesized

during thyroid hormone therapy was 64 grams Augmented albumin synthesis thus amounted to about 79 per cent of the increase in albumin degradation In individual subjects the increase in albumin synthesized during thyroid treatment periods of 12 to 22 days ranged from 22 to 128 grams

DISCUSSION

Previous observations in treated myxedema have established alterations in serum protein concentration and distribution Thompson, Thompson, Silveus, and Dailey (8) noted a decrease in serum protein concentration when thyroid hormone was administered to two subjects with myxedema, and Boothby, Sandiford Sandiford, and Slosse (9) observed a negative nitrogen balance following thyroxine administration in myxedema and concluded that extravascular sites were the source of the lost protein Thompson (10) observed a decrease in blood volume in myxedema which returned to normal with replacement therapy and Gibson and Harris (11) noted an increased blood volume in hyperthyroid subjects Schwartz (12) and Lewallen, Rall, Berman, and Hamel (13), employing I^{131} labeled albumin, observed a decrease in extravascular albumin in myxedematous subjects treated with desiccated thyroid The present study is consistent with these observations and indicates further that the reversal of the abnormalities present in myxedema is not simply referable to correction of a metabolic defect due to lack of thyroid hormone but also that similar changes can be induced by excessive amounts of the hormone even in the absence of such a defect This is in accord with the widely held concept that thyroid hormone does not produce any qualitative changes in metabolism but acts as a regulator for the quantitative control of autonomous functions However, the present studies do not rule out the possibility that the changes induced in myxedematous patients and in euthyroid subjects are mediated through qualitatively different mechanisms Since the precise mechanism of action of thyroid substances has not been definitely established, speculation on this point seems unwarranted at present

Of special interest is the observation that, under the influence of thyroid hormone, albumin production by the liver increased to a level which nearly compensated for the increased albumin utilization, as a result of which no appreciable negative albumin balance occurred This is in contrast to previous observations in proteinuric subjects that a decrease in the rate of albumin degradation rather than an increase in the rate of albumin synthesis was the mechanism by which the body generally compensated for the renal losses (2) It was not clear whether the failure to increase the rate of albumin synthesis in these cases represented a pathologic or physiologic limitation, since a protein synthesizing defect in nephrotic proteinuria has previously been suggested (4) However, it has recently been demonstrated that the low serum protein bound iodine levels frequently observed in nephrosis are associated with a diminished rate of metabolism of thyroxin (14) It would then seem that the diminished degradation and limited synthesis of albumin in proteinuria are compatible with normal liver function in a hypometabolic state The maintenance of a low serum albumin concentration in the presence of significant proteinuria actually minimizes protein loss in the urine and consequent depletion of tissue proteins since albumin excretion would be expected to increase with increased albumin concentration, even if there were no rise in the rate of plasma albumin clearance by the kidneys Thus, a decrease in albumin degradation without stimulation of albumin synthesis appears to be an economical means of conserving body protein in the presence of proteinuria Because of the direct stimulation of catabolism, a similar mechanism is not possible in thyrotoxicosis Hence, the body adapts to protein deficit in different ways depending upon the manner in which this deficit is acquired

The present study indicates that, at least under the influence of excess thyroid hormone, the normal liver is able to elaborate increased amounts of serum albumin Whether or not the normal liver can increase its output under euthyroid conditions, assuming the demand is created by increased loss or utilization, cannot be answered by these data Whipple and Madden (15) observed a rapid restitution of serum protein concentration following plasmaphoresis in dogs and attributed this to an increased rate of protein synthesis However since the rate of protein degradation was not studied, the possibility that replenishment of protein

stores was effected by significant slowing of protein catabolism rather than by acceleration of synthesis cannot be excluded

SUMMARY AND CONCLUSIONS

1 Methods are described for the quantitative evaluation of albumin degradation and albumin synthesis under non steady state conditions

2 The distribution and metabolism of albumin-I^{131} were studied in nine subjects before and after the administration of large doses of desiccated thyroid. Clinical and laboratory evidence of hypermetabolism developed during thyroid administration in all subjects

3 There was a decline in total serum protein concentration in all subjects with a fall in both albumin and globulin fractions The total intravascular albumin remained essentially unchanged due to a concomitant increase in plasma volume.

4 The fractional rate and absolute amount of albumin degraded daily increased in all subjects. However augmented albumin synthesis resulted in only a small loss of total exchangeable albumin. This loss was sustained almost entirely by extravascular sites

APPENDIX

Incidental observations

Since there is a paucity of data on experimental hyperthyroidism in human subjects the following observations are recorded. In all subjects the first symptoms of hyperthyroidism appeared in about two weeks Tremor was noted in 7 subjects and heat intolerance in 4 Appetite was noticeably increased in 3 subjects but diminished in 1 patient. None of the subjects developed diarrhea, and only 1 subject noticed an increase in bowel movements. Five of the 9 subjects complained of occipital headache present on arising and lasting for several hours, which is not commonly reported in association with hyperthyroidism. This symptom could not be attributed to hypoglycemia since fasting blood sugar values were normal and the headaches were not relieved by food. There were weight losses of 8 to 19 pounds over the 13 to 25-day periods of thyroid administration in all of the 5 subjects in whom weights were recorded. Blood pressure values remained essentially unaltered during thyroid therapy The resting heart rate increased to 96 beats per minute or more in 8 of 9 subjects. The basal metabolic rate increased from +22 per cent to +49 per cent above the control level with a mean rise of +37 per cent. In three subjects in whom serum protein bound iodine concentrations were obtained, values ranged from 9 micrograms per cent to 14 micrograms per cent during the period of thyroid administration. Control values were not obtained but the normal range in this laboratory is 3.8 to 7.5 micrograms per cent. Total serum cholesterol concentrations were depressed to approximately 60 per cent of the control values and no abnormalities in serum bilirubin or cephalin flocculation were noted in 3 subjects. However in these 3 subjects the thymol turbidity fell from 3.2, 3 4 1.6 to 1 1 2.0 0.5 Shank Hoagland units, respectively

ACKNOWLEDGMENTS

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ERYTHROCYTE PRESERVATION VIII METABOLIC DEGRADATION OF NUCLEOSIDES *IN VITRO* AND *IN VIVO*[1]

By MARION HENNESSEY CLEMENT A. FINCH, AND BEVERLY WESCOTT GABRIO

(From the Department of Medicine University of Washington School of Medicine Seattle Washington and King County Blood Bank)

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It has been shown previously that the addition of inosine to ACD preservative[2] prolongs the effective period of *in vitro* storage of erythrocytes (1) Inosine is utilized by the red cell after a phosphorolytic cleavage to ribose-1-phosphate and hypoxanthine, mediated by a nucleoside phosphorylase (2, 3) Ribose-1 phosphate enters the "aerobic shunt pathway" of glucose metabolism after conversion to ribose-5-phosphate, and a subsequent effect is a generation of ATP[3] and the resultant maintenance of the energy reserve of the red cell (4) Inasmuch as the red cell lacks the enzyme xanthine oxidase (5) it is evident that the other cleavage product hypoxanthine, is not metabolized further but remains in the red cell and the plasma during storage.

The present investigation is concerned with the rate of conversion of inosine to hypoxanthine by the red cells and the ratio of these substances in the plasma of blood stored in ACDI, as well as the *in vivo* metabolism of inosine after infusion Related studies on the nucleosides adenosine and guanosine, will be presented also

METHODS

Human blood was collected in ACD and the nucleoside, dissolved in 0.9 per cent NaCl, was added with sterile precautions

Inosine and adenosine were obtained from Schwarz Laboratories hypoxanthine and guanosine from Nutritional Biochemicals Corporation.

Hemolysis during storage was determined by the measurement of plasma hemoglobin as the pyridine hemochromogen according to the method of Flink and Watson (6)

Paper chromatography (one-dimensional) on Whatman No. 1 filter paper was performed with the following solvent systems isobutyric acid concentrated ammonia water (66 1 33) n butanol water (86 14) and water adjusted to pH 10 with NH_4OH according to the methods described previously (1) After identification of the purine-containing compounds with the use of an ultraviolet light (Mineralite) the materials were eluted from the paper with water and measured spectrophotometrically These substances were estimated also by quantitative densitometry[4] of the paper chromatograms.

The hypoxanthine content of the plasma and red cells was determined enzymatically using xanthine oxidase (7) except that the assays were performed on neutralized PCA filtrates of the various fractions.

Acid filtrates of plasma were prepared in the following way 2 ml. of plasma were added to 2 ml. of cold 0.6 N PCA, mixed well and centrifuged at 4 C for 5 minutes at $15,200 \times g$[5] The precipitate was washed once with 2 ml. of cold 0.3 N PCA and the centrifugation was repeated. The supernatant fluid and the washing were combined and neutralized with cold 20 per cent KOH followed by centrifugation at 4 C for 10 minutes at $15,200 \times g$ The $KClO_4$ precipitate was washed once with 2 ml. of cold distilled water and centrifugation repeated. This second supernatant fluid and the washing were combined for analysis Acid filtrates of the red cells were prepared in essentially the same way Approximately 4 ml. of cells were washed twice with an equal volume of cold 0.9 per cent NaCl each time with centrifugation for 20 minutes at $1,700 \times g$[6] The washings were discarded, since they contained only negligible amounts of ultraviolet absorbing materials.

A 50 per cent cell suspension was prepared with 0.9 per cent NaCl. Hematocrit determinations were made on this cell suspension. One ml of cell suspension was added to 3 ml. of cold 0.6 N PCA, and the neutralization of the acid filtrate proceeded as described above.

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[2] Acid-citrate-dextrose, National Institutes of Health Formula B

[3] The following abbreviations have been used ATP = adenosine triphosphate PCA = perchloric acid ACDI ACDA ACDG = acid-citrate-dextrose, inosine, adenosine or guanosine, respectively

[4] Photovolt densitometer Model 301A, phototube B filter 5265 wave-length 253 mμ.

[5] Twelve thousand rpm International Centrifuge Model PRI high speed attachment radius from bottom of tube equals 9.5 cm.

[6] Three thousand rpm International Centrifuge Model PRI rotor No. 269 radius from center of tube equals 17 cm

Absorption spectra measurements in the region of 230 to 300 mμ were obtained with the use of the Beckman DU spectrophotometer on the PCA filtrates of plasma or red cells Since both hypoxanthine and inosine were present in the plasma of blood stored in ACDI or ACDA (1, 4, 8), the relative amounts of each substance could not be estimated directly from light absorption measurements alone. However, after the amount of hypoxanthine had been determined by means of xanthine oxidase, it was possible from the light absorption data on the plasma filtrates, to correct the extinction values at 249 mμ (peak light absorption of hypoxanthine, millimolar extinction coefficient = 10 5) for hypoxanthine concentration. The readings were also corrected for the light absorption of appropriate control samples, i e, untreated with nucleoside. Thus a value was obtained which was due to inosine. Approximately 400-fold dilutions were made of all samples for spectrophotometric measurement, and the light absorption of the control samples during storage was less than 2 per cent of the nucleoside-treated samples

Uric acid was determined on plasma and urine by the uricase method of Dubbs, Davis, and Adams (9)

RESULTS AND DISCUSSION

Storage of blood in ACDI

The ratio of hypoxanthine to inosine during the storage of blood in ACDI at 4° C was determined in two experiments In the first investigation, only the ratio in the plasma was estimated, while the second study involved the ratio of the two compounds in both plasma and red cell fractions

Experiment 1 Human blood was collected in ACD and divided into two 100-ml aliquots After 24 hours of storage at 4° C, 20 ml of 0.9 per cent NaCl were added to one aliquot, and 20 ml of inosine solution in 0 9 per cent NaCl (1300, μmoles, i e, 3,000, μmoles per 100 ml red cells) were added to the other Analyses were performed subsequently on days 4, 9, 15, 23, 37, and 57

There is progressive uptake of inosine by the red cell (cf, Table I), so that by 57 days there are only 14 μmoles of inosine remaining in the plasma, while 1,286 μmoles have been taken up by the cells Of this amount absorbed, 440 μmoles have returned to the plasma as hypoxanthine, leaving 846 μmoles inside the cells as inosine and hypoxanthine Paper chromatographic experiments revealed that only two ultraviolet absorbing materials, i e, hypoxanthine and inosine, were present in the plasma fractions throughout storage Furthermore, quantitative densitometry of the chromatograms confirmed the ratios of hypoxanthine to inosine given in Table I The enzyme responsible for the phosphorolytic cleavage of the nucleoside, nucleoside phosphorylase, has been shown previously to be in the soluble portion of the red cells (3)

After phosphorolytic cleavage of inosine, the ribose moiety is metabolized further by the red cell during storage (4), while the nitrogenous base, hypoxanthine, remains unchanged As the time of storage progresses, hypoxanthine diffuses from the red cell into the plasma fraction in increasing amounts

Experiment 2 Human blood was collected in ACD and divided into two aliquots 1) 100 ml blood + 20 ml saline, 2) 100 ml blood + 20 ml inosine solution (1,272 μmoles, i e, 3,500 μmoles per 100 ml red cells) Both aliquots were stored at 4° C for 36 days The data are reported in Table II After 36 days of storage there were 742 μmoles of hypoxanthine and 361 μmoles of inosine distributed between the plasma and red cell fractions, or a total of 1,103 μmoles of material This leaves 169 μmoles (13 per cent of the original amount of inosine) unaccounted for This loss of material is unexplained, at present, although paper chromatography indicated the presence of an unknown compound capable of absorbing ultraviolet light in the nucleoside-treated sample which was not present in the control erythrocytes

TABLE I

Plasma hypoxanthine inosine ratio of blood stored in ACDI

	Plasma fraction* †				
Days storage	Inosine (μmoles)	Hypoxanthine (μmoles)	Hypoxanthine Inosine ratio	Inosine absorbed (μmoles)	% Inosine absorbed
1	1,300‡				
4	928	143	0 15	372	29
9	709	241	0 34	591	46
15	538	296	0 55	762	59
23	277	298	1 08	1,023	79
37	150	376	2 51	1,150	89
57	14	440	31 40	1,286	99

* The plasma fraction represents the plasma, ACD, and the saline diluent for inosine added to the 100 ml aliquot of blood (20-ml inosine solution)

† All values have been corrected for the values of the control sample (100 ml aliquot of blood stored in ACD + 20 ml saline)

‡ Thirteen hundred μmoles inosine added after 24 hours' storage

TABLE II

The relationship of hypoxanthine to inosine in plasma and red cells of blood stored in ACDI

Human blood was stored for 36 days in ACD (100 ml. blood + 20 ml. saline) and in ACDI (100 ml blood + 20 ml inosine = 1272 μmoles)

	36 days stored (ACDI)		
	μmoles Is*	μmoles Hx*	Hx:Is ratio
Plasma fraction	330	529	1 6
RBC fraction	31	213	6 9
Total†	361	742	

* Hx = Hypoxanthine Is = Inosine.
† Total recovery of 1,103 μmoles (361 + 742) is 87% of the original amount. All values are corrected for those of the control (ACD)

Calculations of the concentration of hypoxanthine in either plasma or red cells revealed that at 36 days of storage there was an equilibrium established (approximately 6 μmoles hypoxanthine per ml)

Storage of blood in ACDA and ACDG

Similar experiments were carried out on blood stored in ACD with the addition of either 1,200 μmoles of adenosine or 1,300 μmoles of guanosine under conditions identical to those described in Experiment 1

In the study of blood stored in ACDA it was apparent from paper chromatographic analysis that, after 4 days of storage, there was no adenosine in the plasma fraction, and that inosine and hypoxanthine were the only ultraviolet absorbing substances present. In the plasma filtrates throughout storage (*i e* from 4 to 57 days) maximum light absorption occurred between 247 and 249 mμ. By direct measurement, the amounts and ratios of hypoxanthine and inosine were similar to those described for blood stored in ACDI *e g* on days 4 9 15, 23 37 and 57 the ratios of hypoxanthine to inosine in the plasma were 0 09 0.23 0.76 1 60 2.20 and 35.3 respectively Thus it appears as if storage of blood with adenosine is quite similar to storage with inosine except that the enzymatic conversion of adenosine to inosine *via* the adenosine deaminase (10) results in the liberation of ammonia which accumulates in the blood during storage (1) This conversion of adenosine to inosine is complete after

TABLE III

Hemolysis during storage of blood in ACDI and ACDA

Days storage	Mg. hemoglobin per 100 ml. plasma fraction		
	Control*	ACDI*	ACDA*
4	29	14	15
9	37	24	35
15	59	34	42
23	121	56	61
37	292	102	107
57	1,296	362	329

* Control (ACD) = 100 ml. blood + 20 ml saline, ACDI = 100 ml blood + 20 ml. inosine (1 300 μmoles) ACDA = 100 ml. blood + 20 ml adenosine (1,200 μmoles)

one-hour incubation of stored cells with adenosine (1) The above evidence is confirmatory to previous work from this laboratory (4 11) and in accordance with the observations of Rubinstein, Kashket, and Denstedt (8)

The degree of hemolysis in blood stored in ACDI and ACDA is compared in Table III The similarity is apparent, inasmuch as the presence of either nucleoside suppresses hemolysis during storage, although all values are relatively high due to the fact that at each time of sampling the blood was agitated by thorough mixing

Analyses of plasma removed from blood stored in ACDG show that guanine is the purine base liberated from guanosine as a result of phosphorolytic cleavage. Guanine is not degraded further during *in vitro* storage. Studies employing paper chromatography reveal that the increasing concentration of plasma guanine during storage is of the same order of magnitude as the amount of plasma hypoxanthine found in blood stored in ACDI Neither adenosine nor inosine were detected in blood stored in ACDG

The fate of hypoxanthine and guanine after infusion of their ribosides

It was of interest to investigate the metabolism of purine moieties of inosine and guanosine after intravenous administration of these purine nucleosides into human recipients

Two experiments were performed in which normal subjects received about 7 000 μmoles of inosine intravenously in a 500-ml saline infusion This amount of inosine is equivalent to that required for the preservation of one unit of blood

TABLE IV

Serum uric acid after inosine infusion

Experiment 1. Normal male subject (99 Kg.) received intravenously 6,960 μmoles inosine over a period of 1.5 hr.

Experiment 2. Normal male subject (103 Kg.) received intravenously 7,470 μmoles inosine over a period of 1.25 hr.

Time after end of infusion	Mg. uric acid per 100 ml. serum
Expt. 1	
0	6.1
5 min.	8.8
45 min.	9.8
3 hr.	7.4
5 hr.	8.4
Expt. 2	
0	6.1
8 min.	11.5
1 hr.	10.0
3 hr.	8.9
5 hr.	8.7
8 hr.	8.5
27 hr.	6.1

Levels of uric acid in serum and urine were determined at various time intervals after the infusion. A transient rise in serum uric acid was produced soon after the infusion, but the value returned to normal in about 24 hours (Table IV).

Uric acid excretion was measured over a period of about 2 days after the infusion and was compared to the normal excretion levels of the subjects. Approximately 32 per cent of the dose of inosine was excreted as uric acid during a period of 24 hours after the infusion (Table V). Subsequent sampling indicated that an additional 10 per cent of the dose was excreted over the next 12 hours at which time the uric acid levels had returned to normal.

Although these experiments are preliminary in nature, it is probable that the series of events which occur *in vivo* after the infusion of inosine are those shown in equation (*1*).

TABLE V

Uric acid excretion after inosine infusion

Experimental conditions as in Table IV

	μmoles uric acid excretion above normal in 24-hour post infusion period*	% dose of inosine excreted as uric acid in 24 hr.
Expt. 1	2,427	34.9
Expt. 2	2,344	31.4

* Normal urinary uric acid was established in the subjects as 3,993 μmoles per 24 hours.

Guanosine (7,060 μmoles) was administered similarly to a patient with leucopenic leukemia, and the data recorded in Table VI indicate that the metabolic end product of the purine base in this case was the same, *i.e.*, uric acid. It is probable that the phosphorolysis and oxidation proceed according to equation (*2*).

Previous studies on the subcutaneous injection of guanosine have demonstrated increments of uric acid excretion in the urine (12). While neither these previous results nor the data presented in this communication permit any conclusions as to the urinary yield of converted nucleoside, they would appear to be within the range observed after the intravenous administration of uric acid alone, *i.e.*, about 60 per cent (13).

TABLE VI

Serum uric acid after guanosine infusion

Patient (58 Kg.) with leucopenic leukemia received intravenously 7,060 μmoles guanosine over a period of one hour.

Time after end of infusion	Mg. uric acid per 100 ml. serum
0	4.4
10 min.	10.9
2 hr.	11.9
3 hr.	12.0
5 hr.	13.3
12 hr.	10.8
20 hr.	6.5

$$\text{inosine} \xrightarrow[\text{P}_i]{\text{nucleoside phosphorylase}} \text{hypoxanthine} + \text{ribose-1-phosphate};\quad \text{hypoxanthine} \xrightarrow[(\text{O}_2)]{\text{xanthine oxidase}} \text{xanthine} \xrightarrow[(\text{O}_2)]{\text{xanthine oxidase}} \text{uric acid} \tag{1}$$

$$\text{guanosine} \xrightarrow[\text{P}_i]{\text{nucleoside phorphorylase}} \text{guanine} + \text{ribose-1-phosphate};\quad \text{guanine} \xrightarrow[\text{H}_2\text{O}]{\text{guanase}} \text{xanthine} \xrightarrow[(\text{O}_2)]{\text{xanthine oxidase}} \text{uric acid} \tag{2}$$

SUMMARY

After being taken up by erythrocytes inosine undergoes enzymatic phosphorolysis to yield ribose-1 phosphate and hypoxanthine. The hypoxanthine diffuses outward into the plasma during storage until an equilibrium is reached with that inside the cells

Adenosine is converted rapidly to inosine during storage and is utilized through the same metabolic pathway with the resultant increasing concentration of hypoxanthine in the plasma. Guanosine is utilized similarly with the exception that guanine is the purine base which accumulates in the plasma

The intravenous administration of inosine indicates that the hypoxanthine is oxidized further *in vivo* to uric acid Likewise, guanosine infusion results in increased concentrations of uric acid in the blood serum

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EXPERIMENTAL STUDIES OF THE MECHANISMS PRODUCING HYPOCALCEMIA IN HYPERNATREMIC STATES[1]

By LAURENCE FINBERG WITH THE TECHNICAL ASSISTANCE OF EVELYN FLEISHMAN

(From The Pediatric Division of the Baltimore City Hospitals and the Department of Pediatrics of the Johns Hopkins School of Medicine, Baltimore, Md.)

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That hypocalcemia may occur accompanying or following dehydration in infants has been stressed by Rapoport (1, 2). Several possible mechanisms have been suggested to explain this phenomenon. Rapoport considered the hypocalcemia part of the "post-acidotic syndrome" and thus a part of a readjustment phase following treatment. Hyperphosphatemia secondary to renal impairment could contribute to hypocalcemia as could rapid dilution of the extracellular fluid during therapy. A study of infants with hypernatremic dehydration revealed that hypocalcemia often appeared in this condition prior to therapy and that hyperphosphatemia was not a necessary concomitant (3). Further examination of the data suggested that the serum calcium concentration showed a roughly inverse correlation with the serum sodium concentration. Thus hypernatremia *per se* might be a major causative factor of this hypocalcemia. This report deals with an experimental approach to test the latter hypothesis.

METHODS

The experimental plan was to thirst young rats for a period and then inject sodium salts intraperitoneally; subsequently the animals were to be bled and analyses performed on serum and carcass for electrolytes.

The animals used in the experiments were male albino rats of the Wistar strain weighing between 100 and 200 grams and fed a standard diet. The composition of the diet was: casein, 250 gm.; corn starch, 510 gm.; vegetable oil, 149.5 gm.; Brewer's yeast, 50 gm.; U.S.P. salt mixture No. 14, 40 gm.; and percomorph liver oil, 0.5 gm. The low potassium diet used in some of the experiments was obtained from Nutritional Biochemicals Corporation.

The intraperitoneal solutions injected (100 ml. per Kg.) contained the following concentrations of ions in mEq. per L. (The designations used below will be continued in the text.)

		Na	Cl	HCO_3	K
Isotonic	Na	150	120	30	
Hypertonic	Na	300	240	60	
Hypertonic	Na + K	300	275	75	50

The rats were placed in metabolism cages for urine collection. Except where specified otherwise, the rats were thirsted 24 hours prior to injection and thereafter. A weighed amount of food was left available but it became apparent that the thirsting animals would not eat whether or not they received injections. The animals were sacrificed 24 to 48 hours following injection after anesthetizing with intraperitoneal sodium pentobarbital (50 mgm. per Kg.) and obtaining blood from the abdominal aorta.

Serum was immediately separated from the red cells after clotting and chemical analyses subsequently performed. The chemical methods are those previously reported from this laboratory (3, 4). Sera and urines were analyzed for Na, Ca, Cl, K, Urea N, and osmolarity. The latter determination was made using the Fiske osmometer. In addition P, protein, CO_2 content, and water determinations were performed on the serum. Calcium determinations throughout this study were performed by the method of Harrison and Harrison (5). Carcass analyses were performed by drying the skinned carcass to constant weight and then grinding the dried skin and carcass.

The resultant material was thoroughly mixed and duplicate aliquots were analyzed following ether extraction of the fat. Sodium and potassium were determined by flame photometry after dry ashing. Calculations of the extracellular concentrations of ions were made by correcting for serum water content and by multiplying cation and dividing anion concentrations by the Donnan factor of 0.96. The carcass content of Na, K, and Cl are expressed as mEq. per 100 gm. of fat free solids. Tissue nitrogen is expressed as gm. per 100 gm. of fat free solids. The chloride space (used as an approximation of extracellular fluid) is calculated from the total chloride content of the carcass and the extracellular chloride concentration.

In these experiments the intestinal tract and its contents were included in the carcass analyses in all groups. To assess the possible role of the intestinal content five control animals were analyzed after removal of intestines and compared to five animals simultaneously analyzed

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TABLE I

Results of analyses of sera and carcasses following intraperitoneal injection of hypertonic Na solution into thirsting animals

Serum Ca (mg./100 ml.)	ECF Na (mEq./L.)	Chloride space (ml./100 gm. f.f.s.)	Carcass Na (mEq./100 gm. f.f.s.)	Carcass K (mEq./100 gm. f.f.s.)	Carcass K/N (mEq./gm.)	Carcass Na/K (mEq./mEq.)
7 4	166	119	30.2	25.3	1 82	1.20
7.5	170	125	29 4	28 4	1 72	1 04
8 8	159	126	25 4	23 0	1.81	1 10
8.6	160	126	26.3	21.8	1 93	1.21
7 8	189	128	27 8	20 4	1 47	1 36
8.3	171	96 3	24 7	24 6	1.57	1 01
7 0	174	101	24 7	23 8	1.61	1 04
7 9	180	100	25 4	24 0	1.64	1 06
7 9	161	119	24 4	23.2	1 91	1 05
8 4	155	132	25 9	23 1	1 65	1 12
8.5	176	121	27 8	22 9	1 62	1.21
Average of 16 thirsting control animals						
10.3 ±0.21	147 ±5 1	116 ±3 0	21 4 ±0 22	26 8 ±0.26	2.22 ±0 05	0 81 ±0 05*

* Standard error of the mean

in the usual fashion. No significant differences were found for Na, K, Cl, or N content between the two groups. It should also be pointed out that the thirsting rats in the experiments cited had very little intestinal content when sacrificed.

RESULTS

Behavior changes in the thirsting rats were observed though they were not striking The animals became somewhat withdrawn and lethargic but were hyper irritable to stimuli One animal of the sodium loaded group had some muscle twitchings which simulated tetany but neither the hypocalcemia nor the other chemical derangements in this animal were more pronounced than in others studied Animals receiving the low potassium diet failed to grow and appeared particularly listless and sick after intraperitoneal injection. In general the changes in behavior observed were not consistently different between thirsting controls and thirsting injected animals hence such changes seemed largely attributable to thirsting alone

Hypocalcemia could be regularly reproduced by the intraperitoneal injection of hypertonic sodium solution into thirsting rats The thirsting state was chosen because in preliminary experiments with similar loads where the animals could drink after injection neither sodium excess nor hypocalcemia could be induced The time of bleeding is of some importance in detecting hypocalcemia induced in this manner Animals sacrificed 12 hours following injection did not have hypocalcemia nor did a few surviving rats who were examined at more than 72 hours post injection. Hence the animals discussed herein were all examined between 24 and 48 hours post injection. The pertinent data on eleven such animals with carcass analyses are presented in Table I and contrasted to the average values obtained from 16 uninjected thirsting control animals Though all of the injected animals are hypocalcemic the degree of hypocalcemia does not correlate with the degree of hypernatremia or of body sodium increase Table II contrasts animals injected with the hypertonic Na solution with three types of controls uninjected receiving water ad libitum, uninjected and thirsting and thirsting animals injected with an equivalent volume of isotonic Na solution The mean serum calcium values in the three control groups are in the normal range. There is no overlapping of calcium levels between

TABLE II

Effect of injection of hypertonic Na solutions (100 ml Kg) on serum Ca

Number of animals	Solution	Thirst hours	Mean serum Ca (mg./100 ml)
20	None	None	10.2 ± 0 14*
12	None	48 to 72	10.3 ± 0.22
6	Isotonic Na (150 mEq /L.)	48 to 72	10.2 ± 0.23
27	Hypertonic Na (300 mEq /L.)	48 to 72	7.5 ± 0.38

Standard error of the mean.

the hypertonic Na group and the other three and all of the calcium values are less than 9 mg per 100 ml in the group of experimental animals injected with hypertonic sodium solution Total serum protein concentrations showed no differences among the various groups, the overall range was from 5.74 to 7.78 gm per 100 ml with an average of 6 49 gm per 100 ml These values did not differ significantly from unthirsted rats

Thirsted but uninjected animals lost weight in the order of magnitude of 10 per cent of their initial weight and had very scanty (usually less than 1 5 ml) urine output The animals injected with hypertonic solutions showed slight but variable weight loss at 24 hours (1 0 to 6 0 per cent of initial weight) and put out urine volumes from 3 7 to 9 0 ml in the first 24 hrs When the experiments were carried to 48 hours the weight losses in the injected group approached those of the thirsted controls though the second 24-hour urine output diminished sharply in amount The serum urea N concentration rose in all thirsted animals to about 40 mg per 100 ml and this concentration was roughly proportional to the length of the thirst No intergroup differences were noted Osmolarity determinations carried out on the sera paralleled the sodium concentration in these experiments and appear to add nothing to the data tabulated herein Urine osmolarity determinations revealed the remarkable ability of the rat kidney to excrete a concentrated urine up to values to 3000 mOsm per L Among the animals injected with hypertonic solutions no inter-group differences in osmolarity were noted including the potassium deficient animals

The extracellular sodium concentration in the experimental group was sometimes only slightly increased above control values The carcass analyses however make it apparent that total body Na is consistently increased in these animals whereas in some control animals which have slight hypernatremia but without increased Na content, no hypocalcemia occurred The mean carcass sodium in the experimental animals is 26 6 mEq per 100 gm f f s in contrast to a mean of 21 4 mEq per 100 gm f f s in the controls The chloride space calculations showed no significant differences between hypocalcemic animals and controls A third observation from the carcass data indicated a considerable potassium deficit in the sodium loaded hypocalcemic animals The first two lines of Table III summarize these data Control and experimental values for K/N ratios are given to indicate the extent of the potassium deficits The data suggest that the relative quantities of total body sodium and potassium might be important in determining the observed hypocalcemia The Na/K ratio which expresses this relationship increased from 0 81 in the controls to 1 13 in the experimental animals This rise is the result of combined increase of sodium and decrease of potassium

To test whether potassium deficiency alone might account for the hypocalcemia, rats were made potassium deficient by feeding a virtually potassium free diet for 21 days This diet which

TABLE III

Comparison of mean serum and carcass analyses on controls, hypertonic Na loads, Na + K loads, K deficiency, and K deficiency plus hypertonic Na loads

Animals (No)	Serum Ca (mg /100 ml)	ECF Na (mEq /L)	Chloride space (ml /100 gm f.f.s)	K/N (mEq./gm)	Na/K (mEq /mEq)
Controls (16)	10 3 ± 0 21	147 ± 1 0	116 ± 3	2 22 ± 0 05	0 81 ± 0 05*
Na load— 300 mEq /L 100 ml /Kg (11)	8 0 ± 0 17	169 ± 2 5	117 ± 3 8	1 69 ± 0 04	1 13 ± 0 03
Na + K loaded (7)	10 0 ± 0 25	160 ± 1 1	120 ± 3 4	2 38 ± 0 04	0 86 ± 0 02
K deficient (6)	10 0 ± 0 06	147 ± 1 4	120 ± 3 1	1 75 ± 0 04	0 86 ± 0 03
K deficient plus Na load (8)	7 6 ± 0 12	172 ± 1 9	116 ± 3 3	1 43 ± 0 05	1 43 ± 0 05

* S E M

was also low in magnesium content contained 170 mEq of sodium per Kg The animals were thirsted for 24 hours and half the group were injected with the hypertonic Na solution. The last two sections of Table III compare these two groups of animals The uninjected potassium deficient animals were not hypocalcemic, though the chloride spaces were on the average slightly greater than in the injected animals The ratios of K/N showed the expected decrease The Na/K ratios were only slightly increased over control animals fed the standard diet (Tables I and III) which may be due to the low magnesium content and the relatively low sodium content of the potassium deficient diet Cotlove, Holliday, Schwartz and Wallace (6) found that when rats were fed a diet deficient in magnesium intracellular potassium deficit was not accompanied by an increased intracellular sodium such as occurs on a low potassium, high sodium diet When the intraperitoneal hypertonic Na solutions were given to the potassium deficient animals hypernatremia and hypocalcemia occurred with concentrations of calcium uniformly less than 9 mg per 100 ml. there was a further striking reduction in K/N ratio, and finally the Na/K ratio showed the expected marked increase The experiment indicated that potassium deficiency without sodium excess was not responsible for the observed hypocalcemia.

To test whether sodium excess without potassium deficiency would result in hypocalcemia another experiment was done in which potassium was added to the sodium loading solution Seven rats receiving the stock diet were thirsted and injected with the hypertonic Na + K solution The second and third lines of Table III compare the results in these animals with those in which the hypertonic Na solution was given. The animals given added potassium were hypernatremic but not hypocalcemic, there were no significant differences in the chloride spaces between the two groups the animals receiving Na + K had normal or increased K/N ratios and the Na/K ratio is nearly that of the controls despite an absolute increase in carcass sodium The average value for this sodium was 28.5 mEq per 100 gm fat free solids a clear cut increase over the control value of 21 4 in Table I

No pH determinations were done in these experiments however, CO_2 content determinations showed no differences between thirsted controls and the hypertonic Na loaded animals Values ranged from 16 to 20 mEq per L. except in animals which had received the potassium free diet. In the latter animals CO_2 levels ranged from 25.8 to 31 4 mEq per L. again with no differences between the hypertonic Na group and the 'controls'

The influence of the serum phosphate concentration may be evaluated by the figure in which the concentration of serum calcium is plotted against the serum phosphorus level for these groups of animals hypertonic Na loaded, hypertonic Na + K loaded and thirsting uninjected controls As previously indicated the animals of the hypertonic sodium group show serum calcium concentrations which are all below 9 mg per 100 ml. while the others are all greater than this level On the other hand the serum phosphorus concentrations show considerable overlapping indicating it is not the causative factor for the differences noted in calcium

Urine examination showed no quantitative differences in calcium excretion between hypocalcemic animals and control animals in those experiments where the experimental design resulted in similar urinary volume for the periods studied. Stools were scanty in all groups Fecal calcium analyses in a few control and hypocalcemic rats revealed no differences

DISCUSSION

This series of experiments suggests that an increase in body sodium in the rat will produce a temporary hypocalcemia provided there is a concomitant body potassium deficiency, neither an excess of sodium nor a deficiency of potassium occurring independently produced this effect. In these experiments the hypocalcemic effect was seen only when the Na/K ratio of the total body was 1 0 or greater The predisposing effect of potassium deficiency in the development of hypocalcemia in rats following sodium loading has a possible counterpart in infants with hypernatremic dehydration. Rapoport Dodd Clark and Syllm (1) described infants in the recovery phase of diarrheal disease who were hypocalcemic and hypokalemic. A number of the infants with hyper

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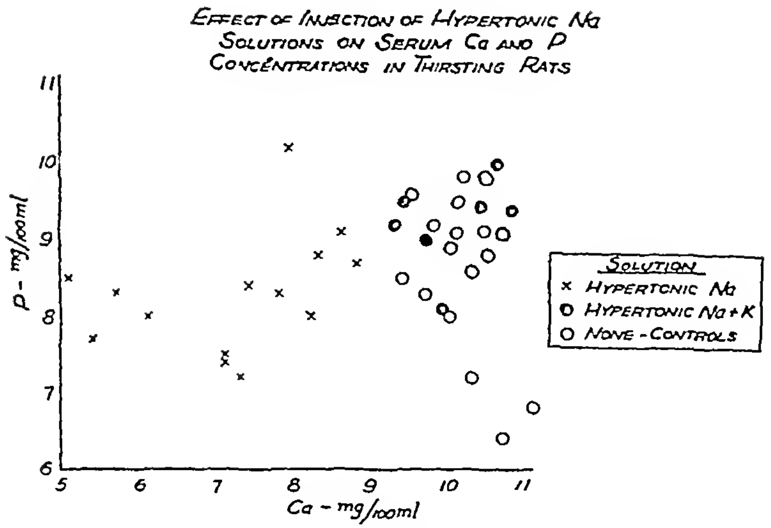

FIGURE 1

natremic dehydration studied by Weil and Wallace (7) as well as some of those studied in this clinic (3) had hypokalemia even during periods of marked urea nitrogen retention

The infant with hypernatremic dehydration does not necessarily have hypocalcemia (3) While most such infants probably often have a reduced body sodium content it is possible that some may actually have sodium contents in excess of normal because of previous excessive dietary or therapeutic intake At present it would be merely speculative to implicate an absolute sodium excess in such infants, or to say that these are the ones who become hypocalcemic Some of the patients have become hypocalcemic after the administration of large amounts of sodium salts Depletion of potassium which was found to be a necessary condition in the experimental animal for the occurrence of hypocalcemia seems a probable part of the usual picture of hypernatremic dehydration in the infant (1, 3, 7) The mechanism whereby the potassium acts is not clear

The concentration of calcium in the extracellular fluid is determined by a number of influences In this study there appears to be no concern with the portion of calcium bound by serum protein since no differences in protein occurred among the various groups of animals Changes in intestinal absorption seem unlikely to be significant since little or no food was ingested by the rats once the thirsting period began Excretion of calcium in the urine showed no differences between the hypocalcemic animals and the others Thus rapid calcium excretion also seems unlikely to be a factor in this study

The data suggest that the effect demonstrated by this study results from an alteration in the equilibrium point of the balance between extracellular calcium and the skeletal calcium The steady state between dissolved calcium and calcium of bone salt probably involves cellular activity of skeletal tissue as well as a physico-chemical equilibrium between solution and solid phases Parathyroid hormone and vitamin D probably influence the cellular activity involved, but the present data are not adequate to show whether the effect might be mediated through a disturbance in those systems Sodium is considered to accumulate on the surfaces of bone crystals and this sodium is known to act as a sort of flexible sodium reservoir under conditions of physiologic disturbances of electrolyte equilibrium (8, 9) Sudden increases in sodium content of bone surface may well play an interfering role in the maintenance of calcium homeostasis No data bearing on this point are contained in the present study Moreover the "protective" role of the potassium would be difficult to explain since the sodium content of the Na + K loaded animals is as high as those loaded with hypertonic Na Additional experiments are needed to further elucidate the mechanisms involved

SUMMARY AND CONCLUSIONS

Thirsting rats injected with hypertonic sodium solution become hypocalcemic concomitant with a rise in total body sodium and a fall in body potassium Neither extracellular dilution nor phosphate retention were important factors in producing this effect. Sodium excess alone or potassium deficiency alone does not result in the hypocalcemia. The total body sodium was equal to or greater than the total body potassium (measured in milliequivalents) in the animals which developed hypocalcemia. It is suggested that the hypocalcemia results from an alteration in equilibrium between extracellular and skeletal calcium

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STUDIES OF RESPIRATORY PHYSIOLOGY IN THE NEWBORN INFANT III MEASUREMENTS OF MECHANICS OF RESPIRATION[1]

By C. D. COOK, J. M. SUTHERLAND,[2] S. SEGAL,[3] R. B. CHERRY, J. MEAD, M. B. McILROY,[4] AND C. A. SMITH

(*From the Boston Lying-in Hospital, the Children's Hospital, the Department of Pediatrics, Harvard Medical School and the Harvard School of Public Health, Boston, Mass.*)

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Considerable attention has recently been focused on the mechanical factors in respiration of normal adults and of those with respiratory abnormalities. This report presents observations on the mechanics of respiration in 23 normal newborn infants and 2 infants critically ill with neonatal respiratory distress. The data are derived from simultaneous measurements of tidal volume and intraesophageal pressure changes.

MATERIAL AND METHODS

The infants, all of whom were born at the Boston Lying-in Hospital, weighed from 2.4 to 3.8 Kg at birth and were from 1 hour to 7 days old at the time of study. History, physical examination and, in most cases, chest x rays were used to determine presence or absence of respiratory distress. On the basis of observation and previously established criteria (1), 18 of the normal infants were considered to have been studied during periods of quiet, resting respiration. In all, 47 studies were made on 28[5] newborn infants ranging from the 18 infants breathing quietly to those who were sick or disturbed by the procedure. The respiratory rates varied from 24 to 136 per minute.

The two infants studied when critically ill and during recovery were diagnosed as having the neonatal respiratory distress syndrome. This syndrome, which is also called the hyaline membrane syndrome or resorption atelectasis, is characterized by a history of premature birth, cesarean section, fetal distress or maternal diabetes and the clinical picture of increasing respiratory difficulty, cyanosis, and frequently typical x-ray findings (2).

The infants were placed in a 65-liter body plethysmograph (Figure 1) with their faces emerging through a pneumatic cuff. Pressure changes within the plethysmograph for an average respiration were approximately 0.3 cm H_2O and were measured by an electrical manometer (3). With a calibrated syringe and pump, breathing was simulated and the pressure changes were calibrated in terms of volume. Although this calibration was performed after the infant was removed, the resulting error was less than 5 per cent and was therefore not taken into consideration in the calculation. Intraesophageal pressure changes as indices of intrapleural pressure changes were measured with a small water-filled polyethylene catheter (internal diameter 1.0 mm.) passed 10 to 11 cm through the nose or mouth into the esophagus and connected to a second manometer[6] calibrated in cm H_2O. When inserted to this distance, the open catheter tip was shown by x-ray of two infants to be at the junction of the middle and upper thirds of the esophagus. Volume and pressure were recorded simultaneously on a direct-writing oscillograph.[6]

Pulmonary compliance was expressed as the ratio of tidal volume to the change in intraesophageal pressure measured between points of no flow, *i.e.*, at the extremes of tidal volume (Figure 2). Respiratory resistance was measured as the ratio of the total pressure change to the corresponding total flow change between points of equal volume approximately midway in inspiration and expiration (Figure 3). This calculation of resistance has provided a satisfactory approximation of the average flow-resistance of the lungs and air passages during the respiratory cycle in adults (4). The average compliances of the individual infants were calculated from 10 to 20 representative respirations and average resistances from 5 to 10 respirations (Table I).

From the simultaneous recordings of pressure and volume, pressure-volume loops for the respiratory cycle were plotted and from 3 to 6 representative breaths a graphic solution of average work done on the lungs per breath was obtained[7] (Figure 4). As indicated in the

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[1] One of a series of studies supported by a research grant from the Association for the Aid of Crippled Children, New York City.

[2] Public Health Service Research Fellow of the National Heart Institute.

[3] Traveling Fellow of the R. Samuel McLaughlin Foundation, Canada.

[4] Traveling Fellow of the British Post Graduate Medical Fellowship.

[5] Although the data are not included in Table I, determinations of work of respiration of one newborn with congenital heart disease and two with borderline respiratory distress are included in Figures 7 and 8 for comparison of the three methods used to calculate pulmonary work.

[6] An electromanometer and the Polyviso made by Sanborn Company, Cambridge, Massachusetts, were used.

[7] As pointed out in the discussion, these measurements do not allow calculation of *total* "work done on the lungs" but do allow an apparently adequate approximation.

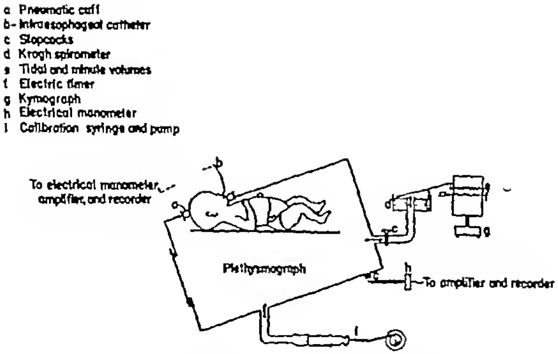

FIG. 1. DIAGRAM OF APPARATUS USED FOR RESPIRATORY STUDIES

With the stopcock to the spirometer closed, the apparatus was used as a pressure plethysmograph. With the stopcock open to the spirometer minute volume was recorded with a photoelectric integrator (1)

diagram this method of measuring work allows separation of pulmonary work into elastic and flow resistive components. For purposes of comparison, pulmonary work for the same respirations was also estimated, using two formulae

1 A simplified formula was suggested by one of the authors (M B McI) as a possibly adequate approximation of work done on the lungs during inspiration and expiration.

Work (in gm cm. per min.) = 0 6 PV

where P = total pressure change in cm H_2O during the respiratory cycle.

V = minute volume in ml

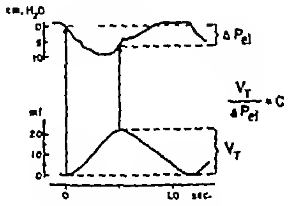

FIG. 2 METHOD OF CALCULATING PULMONARY COMPLIANCE

As shown in this diagrammatic representation of simultaneous pressure and volume recordings, compliance (C) is expressed as the ratio of tidal volume (V_T) to the change in intraesophageal pressure (P_{el}) measured between points of no flow i.e at the extremes of tidal volume.

This formula is based on the fact that, if the intra esophageal pressure is represented by a sine wave purely elastic work would be represented by the formula 0.5 PV (the area of a triangle) purely viscous work by the formula 0 79 PV (the area of an ellipse) and the fact that approximately 70 per cent of pulmonary work in normal adult respiration is elastic (5)[4]

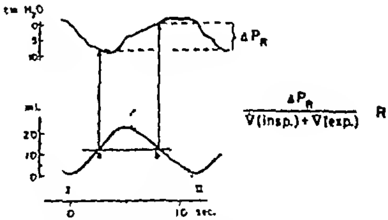

FIG. 3 METHOD OF CALCULATING FLOW RESISTANCE

Respiratory resistance (R) is measured as the ratio of total pressure change (P_R) to the corresponding total flow change ($\dot V_{insp} + \dot V_{exp}$) between points of equal volume (points a and b) Total flow change between points a and b was obtained by measuring the slopes (lines I and II) of the volume curve at these points.

[4] The expression 0 6 PV should not be confused with the expression 0 7 PmaxV used by McIlroy and Eldridge (6) to obtain an approximation to the work of inspiration In that case Pmax was the maximum pressure difference during inspiration In the expression 0 6 PV used in this paper P is the total intrathoracic pressure swing

Since in some infants the introduction of the intra esophageal catheter was followed by an increase in respiratory rate the minute volume rate and tidal volume were obtained in most cases before or after the catheter was in place, utilizing a previously described technique for recording rate and minute volume (1) (see Figure 1) The resting rate and tidal volume were used in the simplified Otis formula for calculating the pulmonary work of the 18 quiet infants. Since these 18 infants had when breathing quietly an average respiratory rate of 38 per minute and an average minute volume of 570 ml. compared to 33 and 550 observed in another group of resting infants of comparable size (1), it was assumed that the average calculated pulmonary work per minute of these 18 infants approximated that of newborn infants in this weight range.

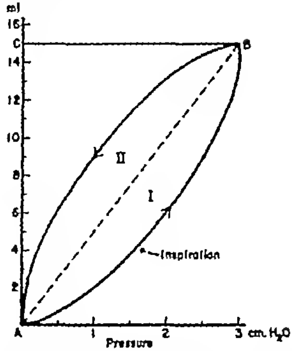

FIG. 4 DIAGRAMMATIC AVERAGE NORMAL PRESSURE VOLUME RESPIRATORY LOOP

Elastic work is represented by area of triangle ABC. Inspiratory and expiratory flow resistive work are represented by areas I and II respectively Assuming that expiration is passive, total pulmonary work is represented by the sum of the elastic work (triangle ABC) plus the inspiratory flow resistive work (area I)

RESULTS

The results of the individual studies on normal infants are presented in Table I The 23 normal infants averaged 3 Kg in weight. The average tidal volume of the quiet infants was 16 ml. (range 9 to 25) Peak flow rates for individual infants averaged 61 ml. per sec. (range 44 to 111 ml. per sec.) during quiet respiration. The average compliance from 38 studies on the 23 normal infants was 4.9 ml per cm H_2O and for the 18 resting infants was essentially the same (5.2 ml per cm H_2O) Although there was considerable variability in both compliance and resistance determinations from breath to breath due to the cardiac component of the pressure recording the standard error of the mean of a series of 10 compliances was only $\pm$ 0 4 ml per cm H_2O The mean resistance was 29 cm. H_2O per L. per sec. (standard error of the mean of a series of 10 = $\pm$ 4 cm H_2O per L per sec.) for these 18 infants and the calculated average work done on the lungs was 1380 gm cm per min In spite of occasional discrepancies the proportion of work done against elastic forces estimated from the pressure volume diagram and from the Otis formula showed on the average a close correlation being 71 and 70 per cent respectively As was expected pulmonary work was greater in the restless infants primarily because of increases in respiratory rates In the present study no significant relation between weight or age and compliance or resistance could be demonstrated presumably because of the relatively small number of infants studied and the narrow weight and age range.

Data from serial observations on two infants severely ill with neonatal respiratory distress are

TABLE II

Data on the mechanics of respiration in two infants with neonatal respiratory distress

No.	Birth wt. (Kg)	Age	IEP* (cm. H_2O)	Compliance (ml./cm H_2O) Mean	Range	Resistance (cm. H_2O/L./sec.) Mean	Range	Work per breath† (gm. cm.)	Work per minute† (gm. cm.)	Per cent elastic work†	Comments
P-40	3 0	2 d	16.3	1.3	1.3–1.5	25	22–29	144	7 900	89	Severely ill
P-40		6 d	4 3	2.5	1.8–3 6	13	5–23	25	1 430	80	Recovering
P 50	3 2	5 hr	20 0	0 7	0 6–0 8	41	0–104‡	107	5 130	71	Severely ill
P-50		2 d	20.8	1 0	0 8–1.3	13	0–27‡	176	[illegible] 400	81	Severely ill
P 50		10 d	7 7	2.5	2.2–3 0	39	18–65	64	3 160	73	Recovering

* IEP is the average of differences between maximal and minimal pressures occurring with each respiratory cycle

† Pulmonary work per breath and per minute and per cent elastic work were estimated directly from the graphic pressure-volume loops for these two sick infants.

‡ Resistance calculated only on inspiration because of grunting expiration

TABLE I

Data from studies of the mechanics of respiration in 23 normal newborn infants

No	Birth wt. *Kg*	Age	Resp rate *per min*	Tidal vol. *ml.*	ΔIEP* *cm H_2O*	Compliance mean *ml/cm H_2O*	Resistance mean *cm H_2O/L/sec.*	Work per breath† *gm cm*	Work per minute† *gm. cm*	% Elastic work†	Comments
P-30	3.7	6 d	39	29	12.6	3.8	79	217	8,460	51	Restless
P-31	3.1	3 d	44	16	2.9	9.1	13	20	885	70	Quiet‡
P-32	2.8	9 hr	41	13	4.4	3.4	28	33	1,350	76	Quiet‡
P-33	3.1	11 hr	42	15	5.4	4.3	24	36	1,490	74	Quiet‡
P-33		6 d	38	19	8.3	3.2	49	84	3,190	67	Restless
P-34	2.4	15 hr	54	13	6.1	2.6	53	53	2,860	62	Quiet—not basal
P-34		38 hr	77	11	6.1	4.4	43	30	2,310	46	Restless
P-34		7 d	40	24	12.4	4.0	70	139	5,560	52	Restless
P-35	3.8	8 hr	70	14	5.0	3.4	25	43	3,010	67	Quiet—not basal
P-38	2.4	10 hr	51	9	5.4	4.6	45	17	840	53	Quiet‡
P-39	3.4	5 d	51	15	8.4	4.1	50	51	2,600	54	Restless
P-41	3.0	2 d	40	15	5.4	5.8	40	34	1,360	57	Quiet‡
P-42	3.5	3 d	31	21	4.5	7.1	42	55	1,700	57	Quiet‡
P-43	3.6	2 hr	28	20	10.4	3.8	131	113	3,160	47	Quiet
P-43		4 hr	48	15	4.1	5.1	18	30	1,445	73	Quiet‡
P-44	2.7	12 hr	38	14	5.5	3.9	45	39	1,480	65	Quiet‡
P-45	2.9	16 hr	24	25	5.3	9.3	43	60	1,440	56	Quiet‡
P-47	2.5	4 hr	35	11	3.3	4.0	24	19	675	78	Quiet‡
P-47		22 hr	136	10	3.2	3.2	25	30	4,080	52	Restless
P-48	2.8	3 hr	42	16	5.6	3.7	21	44	1 850	79	Quiet—not basal
P-48		22 hr	37	18	2.9	8.6	7	22	810	85	Quiet‡
P-49	3.0	2 d	41	18	4.5	7.4	17	31	1,270	71	Restless
P-51	3.3	1 hr	44	13	7.7	2.2	7	60	2,635	64	Quiet‡
P-51		23 hr	66	12	5.3	3.7	24	29	1,910	67	Restless
P-53	3.5	8 hr	57	14	4.0	5.4	13	24	1,370	75	Restless
P-53		2 d	49	12	5.2	2.5	36	39	1,925	73	Quiet‡
P-53		3 d	98	28	5.9	6.8	22	126	12,100	46	Very restless
P-53		6 d	53	15	4.6	4.6	32	40	2,120	61	Restless
P-54	3.1	21 hr	29	21	3.9	7.9	13	35	1,020	80	Quiet
P-54		2 d	25	19	5.2	6.9	21	34	850	77	Quiet‡
P-54		6 d	27	24	5.5	7.1	37	64	1,700	63	Quiet
P-55	3.5	2 d	64	13	6.7	5.9	75	48	3,070	30	Restless
P-55		3 d	35	19	5.3	4.9	35	55	1,925	67	Quiet‡
P-56	2.9	6 hr	30	16	7.0	3.4	19	44	1,310	86	Quiet‡
P-56		30 hr	37	15	9.8	3.3	80	62	2,300	55	Restless
P 56		3 d	51	15	5.5	3.1	41	56	2,840	65	Quiet—not basal
P-57	3.1	13 hr	31	18	5.8	4.8	39	50	1,545	68	Quiet‡
P-60	2.8	6 d	31	15	5.4	3.9	33	38	1,190	75	Quiet‡
Ave all obs	3.1					4.9					
Ave 18 quiet infants	3.0		38	16	5.0	5.2 (S E ±0.4)§	29 (S E ±4)§	38	1,380	70	

* ΔIEP is the average of differences between maximal and minimal pressures occurring with each respiratory cycle during periods of quiet breathing

† Work per breath and per minute and per cent of work against elastic forces have been calculated from the simplified formula of Otis, Fenn, and Rahn (7)

‡ These 18 studies on 18 different infants were used for obtaining average values for quiet respiration

§ S E = standard error of the mean of a series of 10 individual compliances or resistances The standard deviation of a single determination was ±1.28 ml per cm H_2O for compliance and ±11 cm H_2O per L per sec for resistance These large individual variations are apparently due to the artifact in pressure recording introduced by the cardiac impulse

2 Work was also calculated by substituting the determined elastic and resistive factors and tidal volume and respiratory rate in the formula of Otis, Fenn, and Rahn (7) In doing this, it was necessary to assume that the second order resistive factors were negligible. Thus the formula actually used was

Work (in gm cm per min)

$$= \tfrac{1}{2} f K_{el} (V_T)^2 + \tfrac{1}{4} K_r \pi^2 f^2 (V_T)^2,$$

where $K_{el} = \dfrac{1}{\text{compliance}}$ with compliance expressed as ml per cm H_2O

V_T = tidal volume in ml

f = breaths per min

K_r = resistance in cm H_2O per ml per min

Since in some infants the introduction of the intra esophageal catheter was followed by an increase in respiratory rate, the minute volume rate and tidal volume were obtained in most cases before or after the catheter was in place, utilizing a previously described technique for recording rate and minute volume (1) (see Figure 1) The resting rate and tidal volume were used in the simplified Otis formula for calculating the pulmonary work of the 18 quiet infants Since these 18 infants had when breathing quietly an average respiratory rate of 38 per minute and an average minute volume of 570 ml compared to 33 and 550 observed in another group of resting infants of comparable size (1) it was assumed that the average calculated pulmonary work per minute of these 18 infants approximated that of newborn infants in this weight range.

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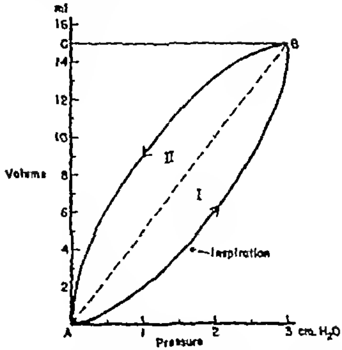

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Elastic work is represented by area of triangle ABC. Inspiratory and expiratory flow resistive work are represented by areas I and II, respectively Assuming that expiration is passive, total pulmonary work is represented by the sum of the elastic work (triangle ABC) plus the inspiratory flow resistive work (area I)

TABLE II

Data on the mechanics of respiration in two infants with neonatal respiratory distress

No.	Birth wt. (Kg)	Age	IEP* (cm H_2O)	Compliance (ml./cm. H_2O) Mean	Compliance Range	Resistance (cm. H_2O/L./sec.) Mean	Resistance Range	Work per breath† (gm cm.)	Work per minute† (gm cm.)	Per cent elastic work†	Comments
P-40	3.0	2 d	16.3	1.3	1.3–1.5	25	22–29	144	7 900	89	Severely ill
P-40		6 d	4.3	2.5	1 8–3 6	13	5–23	25	1 430	80	Recovering
P 50	3.2	5 hr	20.0	0 7	0 6–0.8	41	0–104‡	107	5 130	71	Severely ill
P 50		2 d	20 8	1 0	0 8–1.3	13	0–27‡	176	7 400	84	Severely ill
P 50		10 d	7 7	2.5	2.2–3 0	39	18–65	64	3 160	73	Recovering

* IEP is the average of differences between maximal and minimal pressures occurring with each respiratory cycle.
† Pulmonary work per breath and per minute and per cent elastic work were estimated directly from the graphic pressure-volume loops for these two sick infants.
‡ Resistance calculated only on inspiration because of grunting expiration

result of the decreased compliance is the inability of these infants to achieve more than approximately half of normal "crying vital capacity" (20, 21) The demonstration of a marked increase in the work of respiration in neonatal respiratory distress supports the clinical impression that these infants frequently die of exhaustion and indicates that, until this condition can be prevented or specifically treated, therapy should, at least in part, be directly toward support of respiratory efforts

SUMMARY

In summary, 43 observations on the mechanics of respiration in 23 normal newborn infants and 2 infants with respiratory distress have been reported The resistance for an average 3-Kg infant breathing quietly was found to be 29 cm H_2O per L per sec. and the average compliance 5.2 ml per cm H_2O The resting pulmonary work for such an infant was approximately 1,400 gm cm per minute or 1 per cent of basal metabolism In addition, it was shown that three methods of calculating pulmonary work correlated well Finally, it was demonstrated that infants with neonatal respiratory distress have a marked decrease in compliance, and a striking increase in the work of respiration

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PHYSICO-CHEMICAL AND IMMUNOLOGIC STUDIES ON MACROGLOBULINS [1]

By A H SEHON, L GYENES J GORDON M RICHTER AND B ROSE

(From the Allergy Research Division, McGill University Clinic and the Department of Experimental Medicine, McGill University Montreal Canada)

(Submitted for publication October 8, 1956 accepted November 29 1956)

Normal sera are resolved in the ultracentrifuge into two major peaks with sedimentation constants of the order of 4 5 and 6 5 S [2] and a minor peak of heavy materials amounting to less than 3 per cent of the total proteins with a sedimentation constant of about 20 S

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The amino-acid composition of the macroglobulins has recently been investigated (5–7) The β- and γ-macroglobulins appeared to differ only slightly in their amino-acid content from normal β- and γ-globulins

During the past two years, we have investigated the sera from four patients with macroglobulinemia Their case histories have been reported elsewhere (13) The physico-chemical and immunologic results are reported here

[1] Supported by a grant from the Department of National Health and Welfare, Canada. We are also indebted to Charles E Frosst Co, F W Horner Co Montreal and Eli Lilly Co, Indianapolis for assistance. Presented in part at the meeting of the Federation of American Societies for Experimental Biology in Atlantic City N J., April 1956

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METHODS

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Each of the sera was diluted 16-fold with distilled water. The macroglobulins[3] which precipitated out were centrifuged and the precipitates were washed 3 times with cold distilled water. The precipitates were then dissolved in saline (0.9 per cent NaCl). In attempts at further purification, the macroglobulins of two sera were reprecipitated twice from the saline solution by addition of distilled water. In view of the close similarity of the ultracentrifugal and electrophoretic patterns after the first and third precipitations the macroglobulins of the other two sera were precipitated only once, thus limiting the inherent losses associated with successive reprecipitations. The macroglobulins in saline solution were Seitz filtered into sterile vials and stored at 4° C. After precipitation of the macroglobulins, the supernatants were concentrated to the original volumes of the sera by pervaporation in Visking tubing under sterile conditions. The whole sera, the precipitated macroglobulins and the supernatants were compared by electrophoresis, ultracentrifugation and immunologic methods.

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As shown in Figure 5 the γ-globulins were divided into four sub fractions in an attempt to delimit more precisely the locale of the macroglobulins.

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As a further test for the specific antigenicity of macroglobulins the following experiment was performed (experiment B). To a 3 ml. aliquot of anti M serum, GG was added in sufficiently high concentration to inhibit any precipitation (45 ml of 2.5 per cent solution) between GG and anti M serum. In a series of 12 tubes 1 ml aliquots of this solution were incubated with equal volumes of M in halving dilutions in concentrations varying from 15 mg. per ml. to 8 γ per ml. In a series of control tubes GG was substituted for M. In another experiment the procedure was repeated using, instead of GG, a solution of concentrated serum proteins (20 per cent in saline) isolated from normal human serum by precipitation with ammonium sulphate at 66 per cent saturation.

[4] Batch 88 Squibb Co., obtained through the courtesy of the American National Red Cross, Washington, D. C.

PHYSICO-CHEMICAL AND IMMUNOLOGIC STUDIES ON MACROGLOBULINS [1]

By A. H. SEHON, L. GYENES, J. GORDON, M. RICHTER AND B. ROSE

(From the Allergy Research Division, McGill University Clinic and the Department of Experimental Medicine, McGill University, Montreal, Canada)

(Submitted for publication October 8, 1956; accepted November 29, 1956)

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Agar gel techniques

To determine the minimum number of antigenic moieties in each antigen solution, the degree of cross reactivity of the various systems and the presence of specific antigenic groups or antigens in the macroglobulin fraction, the following agar gel techniques were used.

Oudin tubes were set up for "single diffusion" (19). The antisera were diluted with 3 volumes of a 1 per cent solution made up in saline. All the antigen solutions used in these experiments were made up to 2 per cent in saline. Each antiserum was tested with the 3 antigen solutions, M, NHS, and GG.

The method recently described by Oudin (20) using glass cells with parallel walls was used to determine the antigenic similarities of M, GG and NHS. In these experiments the antisera and antigen solutions were diluted with equal volumes of 2 per cent agar. The concentrations of the GG, M and NHS were 1.8 per cent, 1.5 per cent and 3.5 per cent, respectively. The lower section of the cell was filled with agar solution containing the antiserum, the central section with agar only, and each of the two halves of the upper section with one of the antigens in agar (see Figure 7).

In order to ascertain further whether the macroglobulin fraction contained any specific antigenic moieties not present in normal serum, an experiment similar in principle to experiment B (described above) was devised. A solution of the normal serum proteins was made up to a concentration of 20 per cent in the agar used for the Oudin double diffusion method. This solution, referred to hereafter as AP, was used instead of pure agar in all compartments of the Oudin cell for "double diffusion." Thus a uniform concentration of normal serum proteins was maintained throughout the whole cell. Anti-M serum in AP was placed in the lower compartment and M in AP was placed in the antigen compartment.

In a control cell the upper section was divided into three segments (Figure 8B). Segments I and II were filled with solutions of macroglobulins in AP, the concentrations of M being 0.75 per cent and 0.38 per cent, respectively. Segment III contained only AP solution.

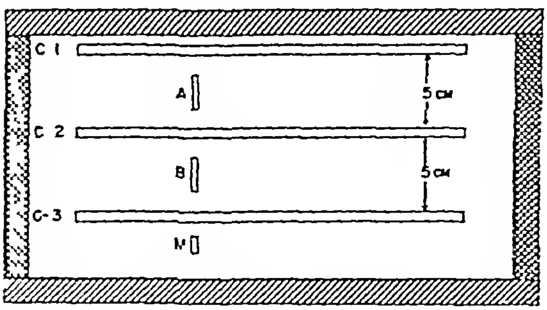

FIG. 1. IMMUNO-ELECTROPHORESIS

A and B represent ditches containing the antigen solution to be separated. C-1, C-2 and C-3 represent channels containing the antisera. M represents the ditch for the marker solution.

Ouchterlony plates (19) with four circular wells symmetrically placed with respect to each other in a Petri dish of 9-cm diameter were used. Two wells, diametrically opposed, were filled with anti-M and anti-GG, and the two other wells with the homologous antigens. The concentrations of the reactants were the same as in the previous experiment.

Immuno-electrophoresis was performed according to the method of Williams and Grabar (21). The glass plates used were 30 cm × 15 cm and were covered with transparent films of agar, as described by Oudin (22). Electrophoresis was carried out in an agar medium (1 per cent agar in veronal buffer at pH 8.6 and ionic strength 0.025). The height of the agar layer was 3 mm. In view of the high degree of electro-osmosis in agar the material to be separated was applied in most experiments, in small ditches (20 mm × 4 mm × 3 mm) 10 cm from the anode (A and B in Figure 1). Two antigen solutions made up in agar[5] were separated simultaneously on each plate and after electrophoresis the antisera in agar were placed into 5 mm wide channels (C) which were cut longitudinally parallel to the direction of electrophoretic separation (Figure 1). To follow visually the electrophoretic separation a "marker" (M) was used. This consisted of normal serum mixed with small amounts of bromphenol blue (travelling slightly ahead of the albumin) and hemoglobin (travelling with the β globulins). The electrophoresis was carried out in a well-insulated box to minimize evaporation. The electrode vessels were identical to those used for paper electrophoresis. The voltage applied across the plate was 30 to 45 volts and the current was about 20 mA. The duration of an experiment was 24 to 30 hours during which time the albumin and γ globulin were separated over a distance of 15 cm. The distribution of the separated proteins was established with the help of a "print," taken as described for starch electrophoresis (18). Immediately after filling channels C-1, C-2 and C-3, the agar plates were covered with glass plates and sealed with silicone grease. The plates were then placed on a level surface at room temperature and observed intermittently for the formation of precipitin bands.

RESULTS

Free electrophoresis

The results of free electrophoresis for the four sera and for the respective supernatants (serum minus macroglobulins) are given in Table I together with the results obtained in this laboratory for normal sera. As can be seen the γ-globulin fractions of all four sera were highly elevated. However, only the first three sera contained appreciable quantities of macroglobulins. The mac-

[5] Two volumes of the antigen solution were mixed with one volume of a 3 per cent agar solution in veronal buffer of ionic strength 0.075.

TABLE I

Free electrophoresis

Case		Albumin	Alpha-1	Alpha 2	Beta	Gamma	Total protein‡	Macro-globulins§
		Relative percentages					Gm. per 100 ml. in whole serum	
			Globulins					
C V (Female)	W	33 1	8 8	19 8	11 8	26.5	7 38	2
N R (Male)	W	16 8	4 8	[illegible] 2	12 [illegible]	58 5	10 68	5
	S	41 4	10 2	5 9	9 6	32 9		
A B (Female)	W	26 8	3 6	7 1	8 9	53 6	16 10	8
	S	61 6	5 0	12 7	12 6	7 1		
L. S (Female)	W	31 3	3 2	7 0	9 2	49 3	12 18	0 [illegible]
	S	34 0	3 4	7 0	9 2	46 4		
Normal† human serum	Male	59 4 ± 3 2	4 7 ± 1 8	8 6 ± 1 4	12 9 ± 1.3	14 3 ± 2 5	8 12 ± 0 59	
	Female	58 4 ± 2.3	5 2 ± 0 7	9 8 ± 1 0	12 4 ± 1 2	14 2 ± 1 8	7 92 ± 0 71	

W—Whole unfractionated serum S—Supernatant (serum minus macroglobulins)
† Average values as found for 22 male and 14 female sera ± one standard deviation
‡ Determined refractometrically
§ Approximate values

roglobulin fraction of each of three sera (C V A B and L S) gave rise to single symmetrical peaks with mobility values of 0 64 1 10 and 0.79 cm per volt per sec. all of which fell below the range of those for normal γ-globulins (1.23 to 1 67 cm² per volt per sec) The macroglobulin fraction of the fourth serum (N.R.) resolved itself into three peaks with mobilities (1 12 3.29 4.70 cm per volt per sec) corresponding to those of γ- β and α_2 globulins The macroglobulin fraction of the serum A.B could not be resolved by free electrophoresis into more than one peak within the pH range 3 6 to 8 6 Figure 2 represents the patterns obtained by free electrophoresis for the whole sera A B and N R and their macroglobulins and supernatants

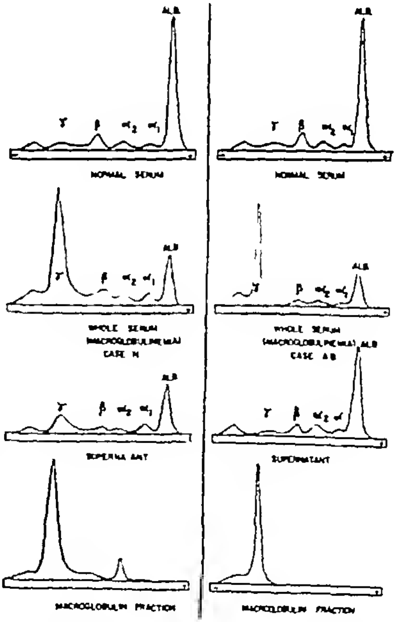

FIG. 2. SEPARATION BY FREE ELECTROPHORESIS

Paper electrophoresis

The results of paper electrophoresis confirmed those obtained by free electrophoresis Staining of the electrophoretograms of the whole sera with fuchsin revealed in addition to the fuchsin stainable bands found in normal sera the presence of material rich in carbohydrate in the γ-globulin region The fuchsin stainable band in the γ-globulin region appeared to be associated with the macroglobulins as evidenced by its presence on the electrophoretograms of the macroglobulin solutions and its absence in the supernatants

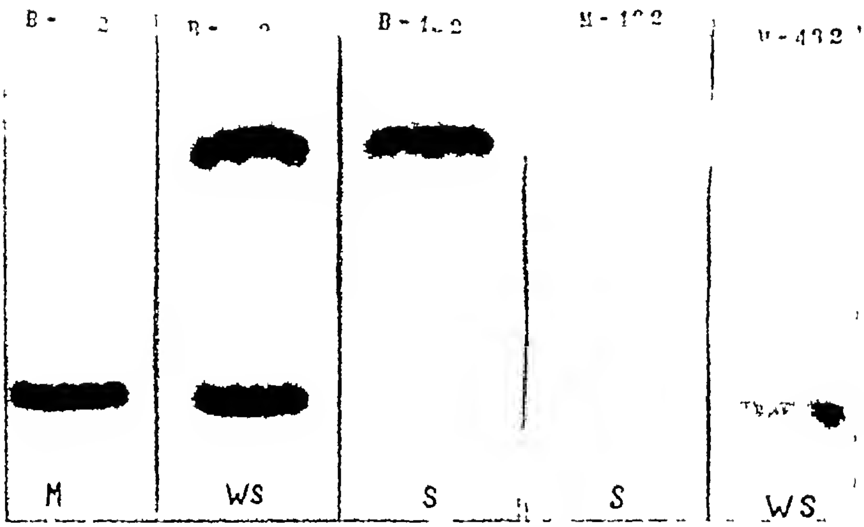

Fig 3 Paper Electrophoresis of Serum A B

WS represents whole serum, M represents macroglobulin fraction, S represents supernatant The three strips on the left are stained with Amido black 10B for proteins while the two on the right are stained with periodic acid Schiff reagent for carbohydrates

Figures 3 and 4 represent the electrophoretograms of the whole sera of A B and N R and their corresponding macroglobulins and supernatants stained for protein and carbohydrate

Separation of A B serum by starch electrophoresis

The resolution of this serum by starch electrophoresis is illustrated in Figure 5 Each of the

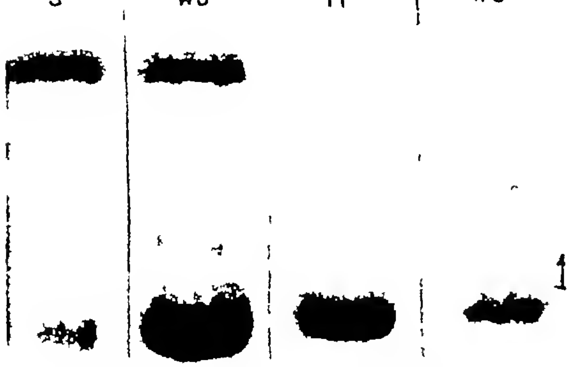

Fig 4 Paper Electrophoresis of Serum N R

WS represents whole serum M represents macroglobulin fraction S represents supernatant. The three strips on the left are stained with Amido-black 10B for proteins, the fourth strip is stained with periodic acid Schiff reagent for carbohydrates

eluted fractions was examined by both paper electrophoresis and ultracentrifugation Four of the eluted fractions, albumin α_1-, α_2- and β-globulins displayed normal migratory and staining properties by paper electrophoresis Ultracentrifugation revealed a rapidly sedimenting material (16 4 S) in low concentration associated with the α_2 fraction The four γ-globulin sub-fractions were all heterogeneous in the ultracentrifuge Three of the latter fractions (B, C, D,) were composed of normal and macroglobulin components Only one fraction (A) appeared to be free of slowly sedi-

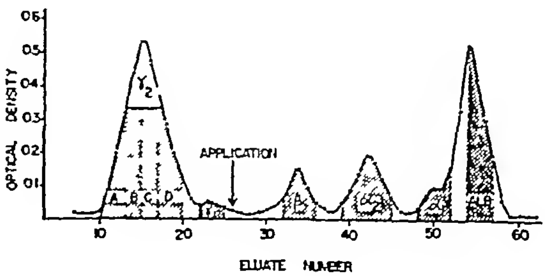

Fig 5 Separation of Serum A B by Starch Electrophoresis

The shaded areas represent fractions eluted from the starch block. A B, C and D represent subfractions of γ-globulins

TABLE II

Sedimentation constants of serum fractions obtained by starch electrophoresis (serum A.B)

Gamma-2								
A	B	C	D	Gamma-1	Beta	Alpha 2	Alpha 1	Albumin
	7 3	6 5	6 5	7 5	4 8	6 7	3 7	4 0
16 4	15 2	15 7	14 6			16 5		
23 2	21 7	22 0	22 2					
	28 8	28 1	28 8					

menting γ-globulin (Table II) The data of Table II would suggest that the macroglobulins migrate at a somewhat slower rate in an electric field at pH 8 6 than do the γ-globulin constituents regularly found in normal serum

Ultracentrifugation

The macroglobulin fractions of the four sera possessed components of high sedimentation constants The results of ultracentrifugal analyses are presented in Table III The macroglobulin fraction of serum N R resolved itself into a broad spectrum of components with sedimentation constants of 3 4 6 0 11 0 12 6 17 7 and 27 5 S By far the greater part of the macroglobulin fraction (82 per cent) was composed of the fast sedimenting material (peaks with S values of 11 to 27 5) The macroglobulins in serum A B (Figure 6) also appeared to be heterogeneous ultracentrifugally possessing sedimentation constants of 16 4 23 3 28 8 S in addition to a small peak with sedimentation constant of 7 4 S These values correspond to the S values determined for the four γ-globulin subfractions isolated by starch electrophoresis The macroglobulins of serum L.S had sedimentation constants of 6 5 10 6 and 15 3 S Sedimentation constants of 6 5 18 5 and 26 0 were calculated for the components in the macroglobulin fraction of serum C.V

TABLE III

Ultracentrifugal analysis of the macroglobulins

Case	Sedimentation constants*	Percentages†
C V	6.5	55 0
	18.5, 26 0	45 0
N R	3 4	5 8
	6 0	11 9
	11 0	11 3
	12 6, 17 7	61.2
	27 5	9 8
A. B	7 4	4 6
	16 4	56.5
	23 3	32 2
	28 8	6 7
L. S	6.5	57.5
	10 6	22 4
	15 3	20 1

* In Svedberg units.
† Relative distribution of the components in the macroglobulin fractions.

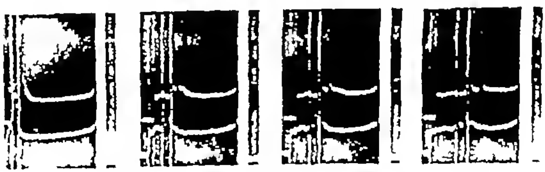

FIG. 6 ULTRACENTRIFUGATION OF SERUM A.B

The lower pattern represents macroglobulins in 1 2 dilution analyzed in a standard cell, the upper pattern represents macroglobulins in 1 3 dilution in a cell provided with a wedge disc. The above frames (from left to right) were photographed at 10 17 22 and 28 min. after the rotor attained full speed of 59,780 r p.m

Fig 7 Double Diffusion Experiments in Oudin Cells with Parallel Walls

M represents macroglobulin fraction of serum A B, GG represents γ globulin, Anti-M represents rabbit anti macroglobulin serum, Anti GG represents rabbit anti-γ-globulin serum, Anti-NHS represents rabbit anti normal human serum

Immunologic results

1 *Precipitin method*—In experiment A precipitation occurred in tubes 2 to 18 in the series of 19 tubes Supernatants from tubes 2 to 18 gave positive ring tests with GG and those from tubes 1 to 12 gave positive ring tests with anti-M serum The supernatants were subsequently absorbed with GG until antibody could no longer be detected against GG However, the supernatants still gave positive ring tests with M thus demonstrating the presence of a precipitating antibody-antigen system specific to M

In experiment B, precipitates were formed only in tubes 5 to 10 of the series of 12 tubes The optimum zone appeared to be in tube 8 Similar results were obtained when the proteins of normal serum were substituted for GG, the optimal zone being observed in tube 9

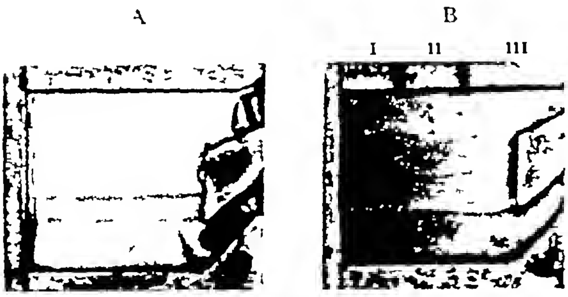

Fig 8 Double Diffusion Experiments in Oudin Cells with Parallel Walls

The cells represent the system of macroglobulins with rabbit anti macroglobulin serum in presence of an excess of normal serum proteins

Agar gel techniques

Precipitin bands were formed in all Oudin tubes indicating thus that M, GG and NHS contained some common antigenic moieties

The results of the experiments using the Oudin cells with parallel walls (Figure 7) demonstrate the extent of antigenic similarity of M and GG[6] In Figure 7A it appears that both M and GG contained at least three identical antigenic moieties In plate 7B seven bands were formed between M and anti-M two of which were common to the GG-anti-M system As revealed in Figure 7C a minimum of four bands was formed only between M and anti-NHS and three additional bands were common to the two systems Figure 8 illustrates the results for the M-anti-M system in agar in the presence of an excess of normal serum proteins As can be seen in Figure 8A one distinct band was produced across the central portion of the cell when the upper section was filled completely with macroglobulin in AP solution This result indicates the presence of at least one specific antigenic moiety in the macroglobulin fraction which is absent from normal serum This conclusion is further supported by the discontinuity of the band

[6] The bands were much more clearly delineated in the agar than in the photographic reproductions

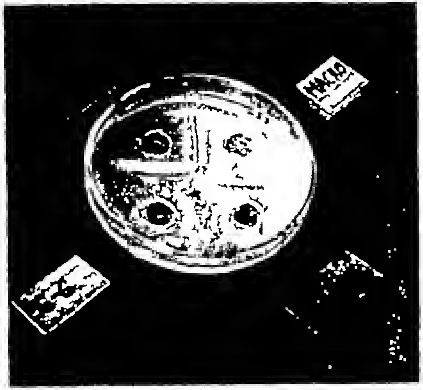

FIG. 9 OUCHTERLONY PLATE

Macro represents macroglobulin fraction of serum A.B., GG represents γ-globulin Anti macro represents rabbit anti macroglobulin serum Anti GG represents rabbit anti-γ globulin serum.

in the region containing only the AP solution in the control experiment (Figure 8B)

The Ouchterlony plate is shown in Figure 9 M versus anti GG gave rise to four bands while each of the homologous antigen antibody systems gave rise to two bands only GG gave only a faint band against anti M

The immuno-electrophoretic results are illustrated in Figures 10 and 11

Electrophoresis of the NHS in agar followed by the application of the anti M, anti NHS and anti GG sera in the longitudinal channels resulted in the formation of a large number of precipitin bands (Figure 10) The anti GG reacted with the electrophoretically separated NHS proteins to yield a long continuous precipitin band extending from the γ-globulin region into the albumin region in addition to two faint bands in the β globulin and albumin regions The long precipitin band was also formed by the NHS anti NHS system but not by the NHS anti M system In all other respects the anti M and anti NHS appeared to form the same number of bands similar in their distribution The absence of bands between anti M and NHS (in Figure 10) and between anti M and GG (in Figure 11) in the region of γ globulins is somewhat perplexing in view of the copious precipitates obtained when anti M was incubated with GG as previously mentioned When M was separated by electrophoresis in the agar and anti M

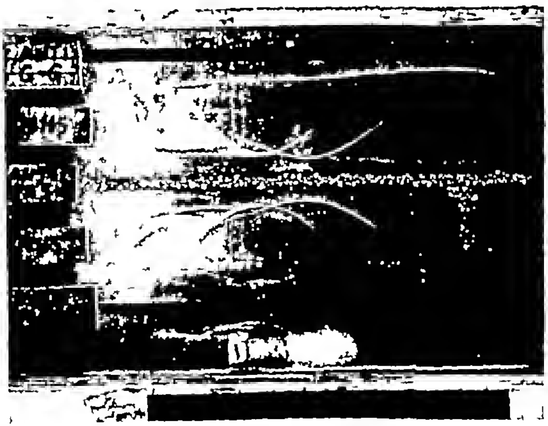

FIG. 10 IMMUNO-ELECTROPHORESIS

NHS represents pooled normal human serum Anti-GG represents rabbit anti-γ-globulin serum Anti NHS represents rabbit anti-normal human serum Anti Macro represents rabbit anti macroglobulin serum.

short bands in addition to a long continuous band extending from the γ-globulin region into the albumin region (Figure 10). This band is most pronounced in the γ-globulin region. This result can be explained in two ways. It may be due to cross-reactivity between GG and all the other proteins normally found in serum. This would imply that the other serum proteins contain some antigenic groups identical to those of γ-globulins. The other explanation would be that γ-globulins are preferentially absorbed onto the agar matrix during their migration by electro-osmosis from the zone of application to their final location on the agar plate.

On the basis of the immuno-electrophoretic results obtained with the GG-anti-GG system (Figure 11) it would appear that the first hypothesis might be ruled out. For as can be seen in Figure 11 a long continuous band similar to the bands obtained with the NHS-anti-NHS or anti-GG system (Figure 10) was also formed between the γ-globulins and anti-GG. This band also extended from the γ-globulin region into the albumin region. However, in view of the contradictory results obtained recently by Slater (24) who claims to have detected some antibody titer associated with β- and α-globulin in addition to the bulk of the antibody residing in γ-globulins, both factors, *viz.*, cross-reactivity of the different serum proteins and absorption, might be partly responsible for the formation of the precipitin band throughout almost all of the protein spectrum. A further point that deserves mention is the reliability of the agar gel techniques in detecting combination between antigen and antibody. Since the anti-M and anti-GG sera formed several clearly delineated bands with either of the two antigens (M or GG) in the Oudin cells (Figure 7), and since copious precipitates were obtained on incubating anti-M with GG in saline, the appearance of only faint lines by immuno-electrophoresis for the anti-M and GG system (Figure 11) is somewhat perplexing. Similarly, only faint bands were formed between anti-M and GG in the single diffusion Oudin tubes and on the Ouchterlony plate (Figure 9). It should be emphasized that although agar gel techniques will, in general, detect trace amounts of antigen or antibody in some systems, in other cases—for reasons which we do not know as yet—almost complete inhibition of precipitin bands may occur.

SUMMARY

Sera from four cases of macroglobulinemia (Waldenstrom syndrome) were investigated by means of zone (paper and starch) and free electrophoresis, ultracentrifugation and immunologic methods (precipitin technique and the Oudin, Ouchterlony and Grabar agar gel technique).

On free electrophoresis the macroglobulin fraction of three sera gave rise to single symmetrical peaks with mobility of a slow moving γ-globulin, while the macroglobulin fraction of the fourth serum was resolved into three peaks with mobilities of α_2-, β- and γ-globulins.

The paper electrophoretograms revealed the presence of material rich in carbohydrate associated with the macroglobulin fractions.

The ultracentrifugal analyses demonstrated that the macroglobulin fractions were heterogeneous, the sedimentation constants of the different constituents varying from 10.6 to 28.8 S.

The immunologic methods indicated the presence of antigenically specific material in the macroglobulin fraction which is absent from normal serum.

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A STUDY IN DOGS OF METHODS SUITABLE FOR ESTIMATING THE RATE OF MYOCARDIAL UPTAKE OF RB^{86} IN MAN, AND THE EFFECT OF L-NOREPINEPHRINE AND PITRESSIN® ON RB^{86} UPTAKE [1]

By W D LOVE AND G E BURCH

(From the Department of Medicine Tulane University School of Medicine and the Charity Hospital of Louisiana at New Orleans La)

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These experiments were performed to test the accuracy of techniques which are suitable for estimating the rate of uptake of Rb^{86} by the myocardium in man In the experimental animal it was possible to compare estimates that were based on external measurements of the type possible in man with the actual myocardial uptake of Rb^{86} determined by direct analysis of the heart after sacrifice The rate of myocardial uptake of Rb^{86} was of interest because factors which influence it, such as the rate of coronary blood flow and the permeability of capillaries and muscle fibers, probably likewise affect the uptake of important metabolites by the heart Since rubidium resembles potassium in its biological behavior, it might be possible to extrapolate to any gross changes in the rate of potassium uptake or concentration occurring with heart disease As a partial test of the relationship between coronary blood flow and Rb^{86} uptake rate in the heart, l-norepinephrine or Pitressin® were administered intravenously to several dogs because of the known effects of these drugs on the coronary blood flow (1, 2) If an estimate of coronary blood flow could be obtained in intact man without catheterization of the coronary sinus, the variations in coronary blood flow in large numbers of normal subjects and patients with various types of cardiac disease could be studied Rubidium^{86}, which has a 1 1 mev gamma emission and a T½ of 19 5 days, has been used as if it were a tracer of potassium since the 12 4-hour T½ of K^{42} makes its use difficult

Rubidium^{86} is not actually a tracer of potassium (3) However, rubidium resembles potassium chemically, and has biologic effects on the heart similar to those produced by potassium (4–7) These two elements are partitioned between the myocardium and plasma in almost identical ratios and the rates of uptake of each by the various organs of the dog are qualitatively similar (8) The rates of uptake of K^{42} and Rb^{86} by the human erythrocyte *in vitro* have been found to be very nearly the same (9) The processes involved in bringing about uptake of the two elements seem to be similar in this type of cell at least, since factors reducing K^{42} uptake, such as cooling, increase in plasma potassium concentration, and the addition of iodoacetate to the plasma, produce a proportional reduction in Rb^{86} uptake (10)

The general procedure in these experiments was to maintain a nearly constant concentration of Rb^{86} in arterial blood by the continuous injection of isotope, usually for 30 minutes, while an indication of the rise of myocardial Rb^{86} concentration was obtained from a collimated, recording scintillation ratemeter placed over the precordium From these data the turnover rate of myocardial potassium, which is defined as the fraction of myocardial potassium exchanging with the plasma per minute, and the amount of plasma cleared of Rb^{86} by 100 Gm of myocardium in one minute were calculated using certain simplifying assumptions The reliability of these estimates was evaluated by comparing them with the results of direct analysis of the myocardium after sacrifice

MATERIALS AND METHODS

Mongrel dogs weighing 6 7 to 14 3 Kg (mean 10 7 Kg) were anesthetized with 30 mg sodium pentobarbital per Kg intravenously and taped to a frame so constructed that they could be held securely in a prone position over the precordial monitor Supplementary anesthesia of 30 to 60 mg sodium pentobarbital was occasionally necessary Under fluoroscopic control the frame was adjusted so that the approximate center of

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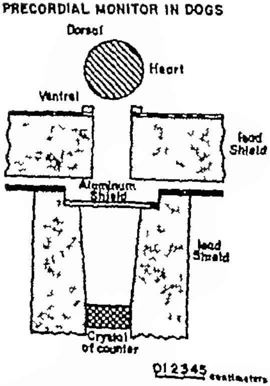

FIG. 1 SCHEMATIC REPRESENTATION OF THE ARRANGEMENT OF THE PRECORDIAL MONITOR IN PRONE, ANESTHETIZED DOGS

the ventricular mass coincided with the vertical axis of the counter crystal After intravenous injection of 100 to 150 mg heparin, frequent recordings of pulse and mean femoral arterial blood pressure were begun using a mercury manometer The mean arterial blood pressure averaged 140 mm. Hg in dogs not receiving drugs and the mean pulse rate was 160 per minute. Blood from the opposite femoral artery was allowed to flow through 300 cm. of 1.7 mm. internal diameter polyethylene tubing and returned to the femoral vein. A drop bottle was interposed, and 60 cm. of this tubing was wrapped around a probe-type Geiger Muller tube attached to a recording ratemeter according to the principle described by Sear (11) Blood flow through the tubing was approximately 15 ml. per min., measurements being made at five-minute intervals by timed collection of 6 ml. blood.

The drugs were administered in 0.85 per cent NaCl solution at an average rate of 4 ml. per min. from a pressurized flask. Flow was regulated with a needle valve and drip bottle. The average dosage of l-norepinephrine was 2.5 μgm. per Kg per min., and of Pitressin®[2] 0.065 pressor units per Kg per min. Administration of the drugs was started 15 minutes before injection of Rb^{86} in order to allow any transient changes in plasma potassium to subside. When Pitressin® was used the initial rise in blood pressure caused by the drug had disappeared before measurements of Rb^{86} uptake were started. The mean pulse rate was 175 per min. and the average mean blood pressure 165 mm. Hg in four dogs receiving l-norepinephrine, while in the six dogs given Pitressin® the average pulse rate was 125 per min. and the average mean blood pressure 145 mm. Hg The hearts of three of four dogs that received l norepinephrine showed varying degrees of intramyocardial hemorrhage, mainly subendocardial, and a decrease in the myocardial potassium concentration. The mean potassium concentration in these dogs was 66.4 mEq. per Kg myocardium, or 20 per cent less than in the control dogs

Precordial monitoring was performed by means of a scintillation ratemeter[3] employing a NaI crystal and a recording galvanometer with a half time of 5 seconds. The geometry shielding and general arrangement of the apparatus are indicated schematically in Figure 1. The precordial radioactivity at the end of 30 minutes of Rb^{86} infusion was approximately 20 times background. Three dogs were sacrificed *in situ* by intravenous injection of 300 mg sodium pentobarbital, and those tissues which were within the field of the counter were removed to determine what portion of the radioactivity recorded over the precordium was actually derived from the heart. This varied from 62 to 72 per cent. Almost one-half of the precordial radioactivity arising outside of the heart was derived from the anterior chest wall while the remaining 15 to 20 per cent originated in the posterior chest wall, lungs, and the portions of the body shielded from the counter In six dogs in which the heart was monitored separately after sacrifice, the amount of radioactivity averaged 70 per cent of the total precordial count.

The injection of Rb^{86} in 0.85 per cent NaCl solution was made into a femoral vein by means of a 50-ml. syringe driven by a variable speed motor The usual Rb^{86} concentration of the injectant was 12 μc. per ml.,

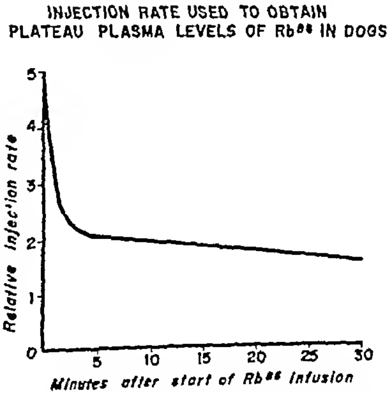

FIG. 2. THE CONTINUOUSLY DECREASING INTRAVENOUS INJECTION RATE USED TO OBTAIN NEAR CONSTANT ARTERIAL PLASMA Rb^{86} CONCENTRATION IN DOGS

[2] Supplied by Parke, Davis & Co.

[3] W S. MacDonald Co. Type 155

35 ml being injected in 30 minutes. Rubidium[86] obtained as Rb_2CO_3 was neutralized with HCl and used no longer than two months after receipt in order to avoid significant contamination with long lived radio elements, such as Cs^{134}. A standard injection rate was derived by calculation from the plasma decay curve of Rb^{86} in dogs (8), with empirical modifications, Figure 2. In some cases the injection rate was altered during the procedure as required by a change in whole blood radioactivity.

Tissue specimens weighing approximately one gram were collected from several parts of the heart, from the lung, liver, and muscle of the pectoral region and spine. These samples were digested in HNO_3 for determination of radioactivity and potassium content by methods previously described (8). Specimens of arterial plasma were obtained at five minute intervals for determination of radioactivity (12) and potassium concentration. In the initial studies plasma potassium concentration was measured using a twenty-fold dilution of plasma. However, all other plasma samples were digested with HNO_3 before dilution, and the earlier determinations were corrected to the probable values which would have been obtained with digested plasma. This correction, which amounted to a 15 per cent increase, was based upon 200 samples measured by both methods.

METHODS AND ANALYSIS OF DATA

Several assumptions were necessary to order to make calculations of myocardial potassium turnover rate and Rb^{86} clearance from the data obtained. As mentioned previously, Rb^{86} was used as if it traced potassium within the myocardium. Therefore, the mass of non-tracer material was represented by the potassium content, which was assumed not to change during the procedure. The rationale of this assumption has been given above. To extend the previous study of the relative concentrations of potassium and exchangeable rubidium in the dog's heart (8), eight animals were sacrificed three to five days after intravenous injection of Rb^{86}, and the potassium and Rb^{86} concentrations of the plasma and myocardium compared. The specific activity of exchangeable rubidium in the heart and plasma have been shown to be nearly equal after 24 hours (8), so that Rb^{86} concentration would indicate relative exchangeable rubidium content. The ratio of the Rb^{86} concentration in the myocardium to that in the plasma divided by the similar ratio for potassium averaged 1.05 ± 0.08, compared with 1.14 in four dogs reported previously. In the calculations this ratio was treated as if it were unity.

The second assumption upon which calculations were based was that the ventricular myocardium constituted a mixed homogeneous compartment exchanging at a single rate with the blood. Although this is clearly not the case, any rapidly exchanging portion containing little potassium, such as the interstitial fluid, would be undetected by the methods used. Furthermore, the results did not indicate the presence of any large slowly ex-

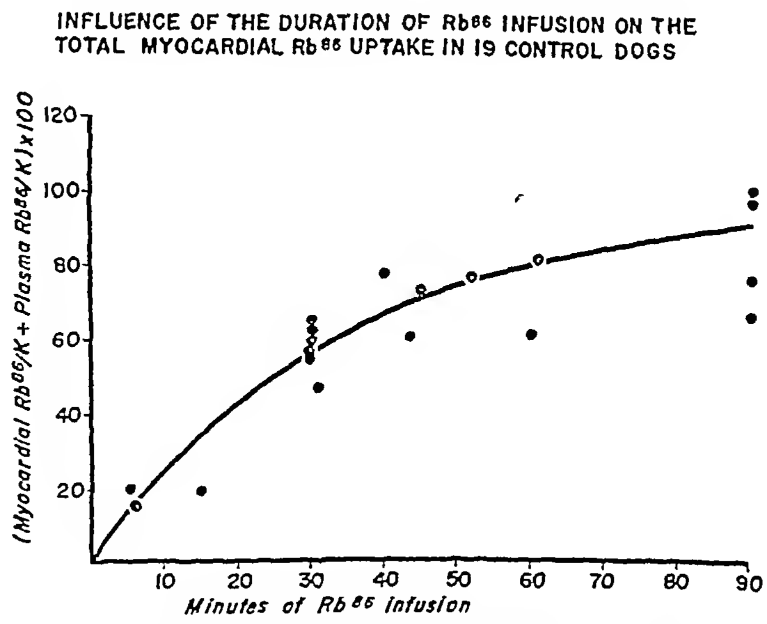

FIG. 3. THE TOTAL Rb^{86} UPTAKE IN 19 CONTROL DOGS DETERMINED AT SACRIFICE 5 TO 90 MINUTES AFTER THE START OF Rb^{86} INFUSION

The curved line represents the time course of Rb^{86} uptake in the hypothetical average dog if the myocardium was homogeneous and exchanged Rb^{86} with the plasma at a single constant rate.

changing components. This is indicated in Figure 3 which shows the time course of the variation in individual dogs about the mean Rb^{86} uptake rate.

In making calculations from data obtained from the intact dog it was further assumed that the precordial monitor reflected the behavior of Rb^{86} concentration in the myocardium. The actual comparison of estimates of myocardial Rb^{86} uptake made on this basis with those obtained from direct analysis of the myocardium served as the test of the usefulness of this assumption. If radioactivity over the heart reflects myocardial radioactivity then the recording of precordial radioactivity has three attributes from which the rate of Rb^{86} uptake by the heart can be estimated. The initial rate of increase of radioactivity over the precordium is related to the initial rate at which plasma is cleared of Rb^{86} by the myocardium, while the level of precordial radioactivity at the end of the infusion of Rb^{86} is related to the maximal myocardial Rb^{86} concentration reached during the procedure and therefore, to the average myocardial clearance of plasma Rb^{86} during the infusion. The amount of curvature of the trace of precordial radioactivity depends in part on the turnover rate of myocardial potassium. These three characteristics have been used to obtain "estimated" rates of plasma clearance and rates of turnover from the type of data obtainable in man and these rates have been compared with their counterparts obtained by direct analysis of the heart at sacrifice. The latter have been referred to as "observed" rates of clearance and rates of turnover

Estimated myocardial potassium turnover rate was obtained by differentiation of the time course of precordial Rb^{86} radioactivity The continuous recording of precordial radioactivity was transcribed and the time course of its slope determined graphically The resulting values were plotted semi logarithmically and a single straight line drawn by inspection which appeared most nearly to represent the data. Usually there was evidence of a rapidly exchanging component during the first five minutes of the Rb^{86} infusion. This portion was ignored in choosing the predominant exchange rate, since it was considered to rise from outside the myocardium. Assuming stable potassium concentrations, the precordial radioactivity curve can be taken to represent the rise of Rb^{86}/K ratio in two compartments exchanging with plasma of constant Rb^{86}/K ratio This may be expressed as

$$Y_t = A(1 - e^{-at}) + B(1 - e^{-bt}) \qquad (1)$$

where

Y_t is the precordial radioactivity at any time t,

A and B are the radioactivities which would be recorded from the two compartments at complete equilibrium, *i.e.*, $t = \infty$

b is the fraction of total non tracer potassium entering or leaving the slowly exchanging portion per minute, and a is the similar fractional exchange rate in the fast exchanging component.

From equation (*1*)

$$\frac{dY_t}{dt} = aAe^{-at} + bBe^{-bt} \qquad (2)$$

The second term of equation (2) obtained graphically as previously described, was considered to represent the variation of Rb^{86}/K ratio in the myocardium.

$$b = \frac{0.69}{T\frac{1}{2}}$$

where $T\frac{1}{2}$ is the time required for the value of the second term to decrease by one-half 100 b is equal to the per cent of the myocardial non tracer entering or leaving the heart per minute.

Estimated initial myocardial Rb^{86} clearance The initial rate of increase of the predominant component of the precordial radioactivity curve was obtained by evaluating the second term of equation (2) at $t = 0$ For each dog the resulting value was plotted against the observed initial myocardial Rb^{86} clearance rate, defined below expressed in units of Rb^{86} cpm. per 100 Gm. myocardium per minute. The resulting empirical relationship (correlation coefficient, $r = +0.95$) was used to convert the observed initial rate of rise of precordial radioactivity in individual dogs to the units of Rb^{86} cpm per 100 Gm. myocardium per minute. The resulting value divided by the average plasma concentration of Rb^{86} was the estimated myocardial Rb^{86} clearance, in units of ml. plasma cleared of Rb^{86} per 100 Gm. myocardium per minute.

Estimated average myocardial Rb^{86} clearance was obtained by taking advantage of the high correlation between final precordial radioactivity and the Rb^{86} concentration of the myocardium at the time of sacrifice ($r = +0.93$) The correlation coefficients between precordial radioactivity and the Rb^{86} concentration of the other organs measured were lung $+0.85$ liver $+0.59$ muscle from anterior chest wall $+0.35$ and back muscle $+0.54$ Despite the variation in ventricular weight from 38 to 89 Gm., there was no consistent increase in precordial radioactivity with increase in heart size. Estimated average myocardial Rb^{86} clearance was defined as

$$\frac{\text{Estimated myocardial } Rb^{86} \div \text{Mean plasma } Rb^{86}}{\text{Duration of } Rb^{86} \text{ infusion}} \qquad (3)$$

Because increasing amounts of Rb^{86} return to the plasma from the heart with increasing duration of Rb^{86} infusion, average clearances of different dogs are not comparable unless measurements are made over the same length of time. As the myocardium approaches equilibrium with the plasma, average clearance becomes an increasingly poor index of initial clearance. Therefore, mean rate of clearance reflects the true rate of myocardial Rb^{86} uptake only when the heart has attained less than approximately 40 per cent of the equilibrium Rb^{86} concentration.

Observed myocardial potassium turnover rate was calculated from the Rb^{86} and potassium concentrations of the myocardium measured at the end of the period of

Rb^{86} infusion using the assumptions listed above. Since the myocardium was assumed to be a single compartment exchanging Rb^{86} and potassium with the plasma at a constant rate, then

$$H = C(1 - e^{-bt}), \qquad (4)$$

where H is the Rb^{86}/K ratio in the myocardium at the time of sacrifice, and C is the Rb^{86}/K ratio in the heart at $t = \infty$ (C is assumed to be equal to the average Rb^{86}/K ratio observed in the plasma) and b and t are as previously defined. The values of H, C, and t were measured directly, and the turnover rate b was determined graphically

Observed initial myocardial Rb^{86} clearance was defined as the amount of plasma at the average concentration of arterial Rb^{86} which would be required to supply the amount of Rb^{86} taken up per minute during a hypothetical moment before any Rb^{86} had begun to return from the myocardium to the plasma. This was obtained from the turnover rate and potassium concentrations as follows

Initial myocardial Rb^{86} clearance

$$= \frac{\text{(b) (Mean myocardial K conc)}}{\text{Mean plasma K conc}} \qquad (5)$$

Observed mean myocardial Rb^{86} clearance was calculated in the same manner as estimated mean myocardial Rb^{86} clearance except that the myocardial Rb^{86} concentration was obtained by direct analysis of the heart muscle.

Changes in plasma Rb^{86}/K ratio occurred during Rb^{86} infusions and caused errors in the indices of Rb^{86} uptake. The influence of such variations was tested by calculating the theoretic changes produced in the time course of myocardial Rb^{86} concentration and the resulting errors in calculations of Rb^{86} uptake. Changes in plasma Rb^{86}/K ratio were assumed to be linear to simplify calculation It is evident from Figure 4 that, in the range of exchange rates and rates of change of plasma Rb^{86}/K ratio actually encountered and investigated, a very significant error may occur in estimations of turnover rate and initial plasma Rb^{86} clearance based on differentiation of the curve of precordial radioactivity However, little error results in the calculations of observed initial myocardial clearance of plasma Rb^{86} and turnover of potassium which depend on the relationship of final Rb^{86}/K ratio in the myocardium to the mean plasma Rb^{86}/K ratio The error in the estimated initial clearance would be greatly reduced if the initial rather than the mean plasma Rb^{86} concentration were used in calculating the initial clearance when a definite progressive change in plasma Rb^{86}/K ratio occurred.

RESULTS

An example of the type of data obtained is given in Table I Figure 5 illustrates the time course of precordial radioactivity in a dog receiving no drug infusion, in one receiving l-norepinephrine,

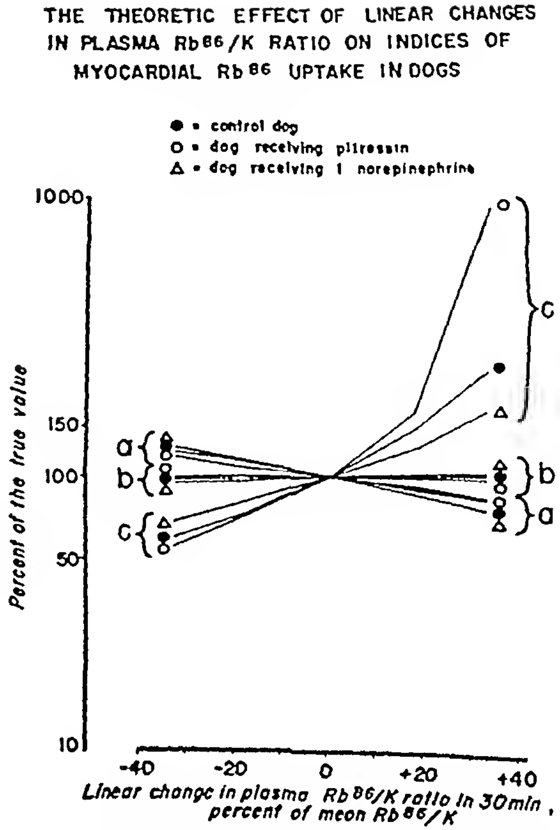

FIG 4 THE THEORETIC EFFECTS OF LINEAR CHANGES IN PLASMA Rb^{86}/K RATIO ON THE CALCULATED INDICES OF MYOCARDIAL Rb^{86} UPTAKE IN DOGS

Since the effect depends on the actual rate of potassium turnover present, calculations were made in the range of turnover rates of myocardial potassium present in the dogs receiving Pitressin® and l norepinephrine, as well as the control dogs Group *a* gives the effects on the calculations of estimated initial myocardial clearance of plasma Rb^{86}, group *b* the effects on calculations of observed initial myocardial clearance of plasma Rb^{86} and turnover of potassium and *c* the effects on calculations of estimated myocardial turnover rate of potassium

and in one receiving Pitressin® The mean plasma Rb^{86} concentration and the relationship between final precordial radioactivity and Rb^{86} concentration of the myocardium were approximately equal in these three dogs

The accuracy of estimations of turnover rate is indicated by Figure 6 Two dogs that received Rb^{86} infusions for less than 7 minutes were omitted since no record of precordial radioactivity was made, and two that were infused with Rb^{86} for 90 minutes were omitted since the myocardial

TABLE 1

Data and calculations from Dog 944 wt 14.3 Kg

Data

	Minutes of Rb^{86} Infusion					
	5	10	15	20	25	30
Rb^{86} *cpm per ml. plasma*	10,900	10,000	11,300	11 000	10,700	10 400
K, *mEq per L plasma*	3.27	3 27	3 24	3 20	3 17	3 17
Mean blood pressure *mm Hg*	113	112	115	118	118	119
Heart rate *per min*	164	164	160	168	168	168

Rb^{86} *cpm per Gm ventricle* (av)	157 000
K, *mEq per Kg ventricle* (av)	77 5
T½ from differentiation of the plot of precordial radioactivity *min*	54
Initial slope of principal component of curve of precordial radioactivity converted to cpm. per Gm ventricle per min	9 980
Final net precordial radioactivity converted to cpm per Gm ventricle	205 000

Calculations

	Observed	Estimated
K turnover of myocardium *per cent per min*	3 1	1.3
Initial myocardial clearance of plasma Rb^{86} *ml. per 100 Gm myocardium per min*	75	93
Mean myocardial clearance of plasma Rb^{86} *ml per 100 Gm myocardium per min*	49	64

Rb^{86}/K ratio at the time of sacrifice exceeded the plasma Rb^{86}/K ratio making it impossible to calculate the rate of turnover Estimated turnover was consistently less than observed turnover in the dogs which did not receive drugs averaging only one-half of the latter value. Figure 6 shows that it was not possible to predict rapid or slow rates of turnover in the control group of dogs Abnormally low rates of turnover were apparent in two of six dogs receiving Pitressin® since

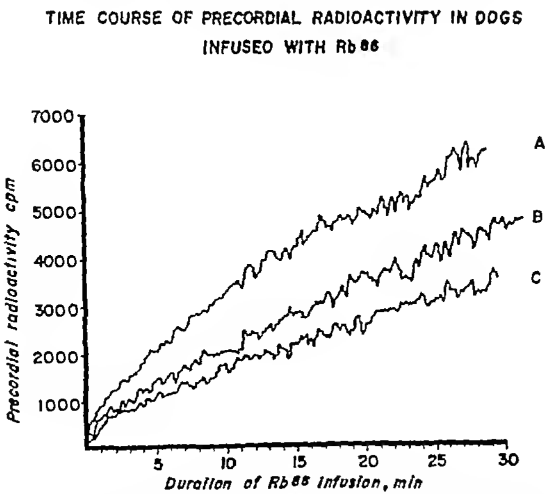

FIG. 5 THE TIME COURSE OF PRECORDIAL RADIOACTIVITY IN A CONTROL DOG (B) AND DOGS INFUSED WITH L NOREPINEPHRINE (A) AND PITRESSIN® (C) DURING A 30 MINUTE PERIOD OF CONTINUOUS RB^{86} ADMINISTRATION

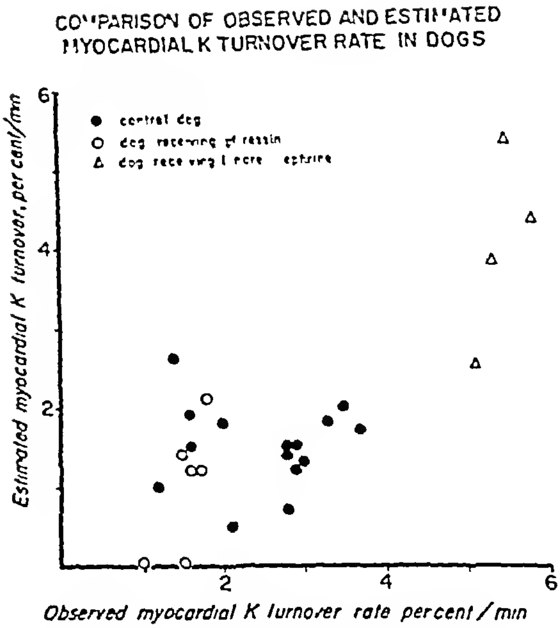

FIG. 6. COMPARISON OF OBSERVED AND ESTIMATED TURNOVER RATE OF MYOCARDIAL POTASSIUM IN DOGS

there was no detectable curvature in the tracing of precordial radioactivity. Abnormally rapid rates of turnover were predicted in all four dogs given l-norepinephrine, although the observed rates of turnover were again somewhat greater than the estimated rates.

The accuracy of estimations of the rates of initial myocardial clearances of plasma Rb^{86} is apparent from Figure 7. The standard error of estimate was 14 ml. plasma, the mean normal clearance being 70 ml. Predicted and observed clearances were necessarily equal overall because of the method of calculation.

The accuracy of estimations of mean rate of clearance in those dogs receiving Rb^{86} for 30 minutes is shown in Figure 8. The standard error of estimate was 10 ml. plasma, the mean control value being 50 ml. There was no consistent difference between the values for estimated and observed mean clearances because an experimentally derived factor was used to obtain the estimated myocardial Rb^{86} concentration from the final level of precordial radiation. In accordance with considerations discussed previously, decreased uptake caused by Pitressin® was detected, whereas the small increase in mean clearance occurring in dogs receiving l-norepinephrine was not.

Factors affecting the accuracy of the three types of estimations included the failure to maintain a constant plasma Rb^{86}/K ratio. In 18 dogs this ratio was not observed to vary by as much as 10

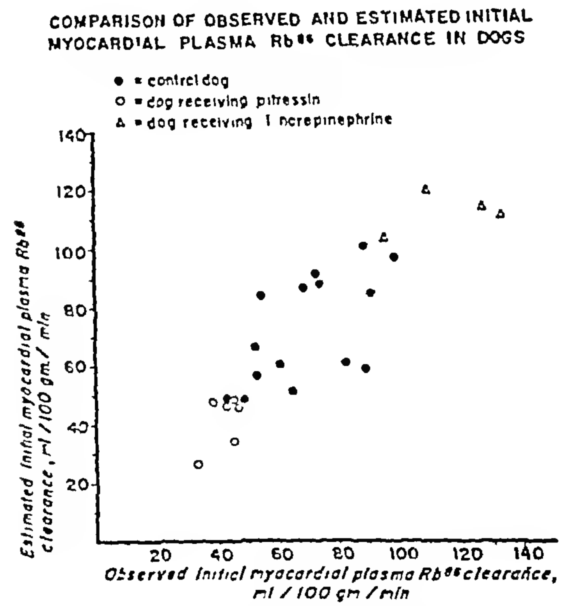

FIG. 7. COMPARISON OF THE VALUES OF OBSERVED AND ESTIMATED INITIAL MYOCARDIAL PLASMA Rb^{86} CLEARANCE IN DOGS

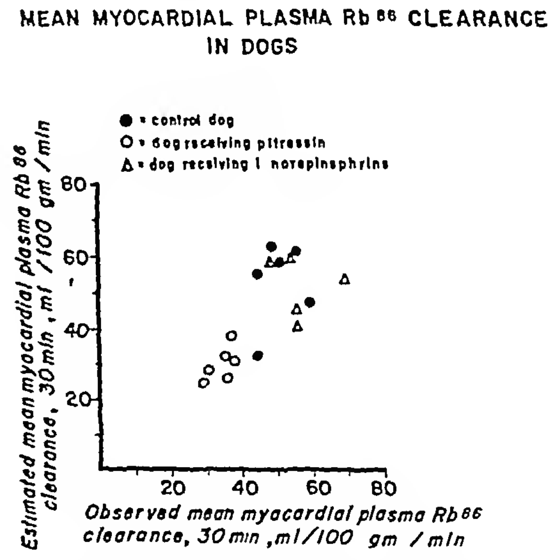

FIG. 8. COMPARISON OF THE VALUES OF OBSERVED AND ESTIMATED MEAN MYOCARDIAL PLASMA Rb^{86} CLEARANCE FOR A 30 MINUTE PERIOD IN THE DOG

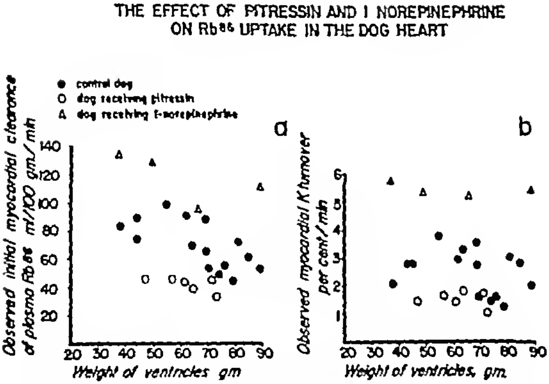

FIG. 9 THE EFFECT OF PITRESSIN® AND L NOREPINEPHRINE ON THE OBSERVED INITIAL MYOCARDIAL CLEARANCE OF PLASMA Rb^{86} (a) AND THE OBSERVED MYOCARDIAL POTASSIUM TURNOVER RATE (b) IN DOGS

per cent of the mean during the Rb^{86} infusion while in the remaining 9 dogs the Rb^{86}/K ratio declined as much as 22 per cent or increased as much as 33 per cent of the mean value In general these changes in plasma Rb^{86}/K ratio were associated with errors in estimated rates of clearance and turnover that were in the expected directions as outlined above, but the quantitative relationships were not consistent enough to justify the application of corrections to the estimated values Some variations in Rb^{86}/K ratio were caused by changes in plasma Rb^{86} concentration but frequently the variations were due to changes in plasma potassium concentration which could not be detected during the experimental procedure.

Variations in the quantitative relationship between the radioactivity observed by monitoring over the precordium and the actual myocardial concentration of Rb^{86} was the major source of error in estimations of the rates of clearance It is apparent that such variations must be related to variations in geometry and heart size and also to differences in the Rb^{86} uptake of the other tissues under the monitor in relation to that of the heart. However attempts to correlate the radioactivity of the lung liver and muscles of the chest wall with variations in the relationship of myocardial Rb^{86} concentration and precordial radioactivity were unsuccessful The presence of important amounts of slowly exchanging tissue under the precordial monitor would result in estimated rates of turnover which would be consistently lower than observed rates as was the case. Dissection of the animals after sacrifice showed that 10 to 20 per cent of the precordial radioactivity was derived from skeletal muscle which is known to exchange slowly (8) In two dogs in which the Rb^{86}/K ratio in the myocardium had nearly reached equilibrium with that of the plasma after 90 minutes of Rb^{86} infusion there was still a continuing rise in precordial radioactivity These slowly exchanging components could not be separated from the myocardial component in the analysis of the time course curve of the first derivative of precordial radioactivity although in theory this might be possible after 90 minutes of infusion of Rb^{86}

The duration of Rb^{86} infusion appeared to influence estimates of the rates of Rb^{86} uptake since infusions of 15 minutes or less provided insufficient data for the separation of rapidly equilibrating components and infusions of long durations *i.e.* 90 minutes accentuated errors caused by slowly exchanging components and variations in the comparative distribution of non-tracer rubidium and potassium

The assumption that the right and left ventricles had the same geometrical relationship to the moni

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DIFFERENCES IN THE RATE OF RB^{86} UPTAKE BY SEVERAL REGIONS OF THE MYOCARDIUM OF CONTROL DOGS AND DOGS RECEIVING L-NOREPINEPHRINE OR PITRESSIN®[1]

By W D LOVE and G E BURCH

(*From the Department of Medicine Tulane University School of Medicine and the Charity Hospital of Louisiana at New Orleans La.*)

(Submitted for publication April 20, 1956 accepted November 29 1956)

The rate at which Rb^{86} enters the myocardium from the blood is determined by the rate of coronary blood flow and by the kinetics of potassium within the myocardium. In preparation for a study of the effects of disease on the rate of myocardial Rb^{86} uptake in man, a trial of methods was made in a series of dogs During this study the rate of Rb^{86} uptake in several regions of the heart of control dogs was measured and the effects of l norepinephrine and Pitressin® on differences in regional Rb^{86} uptake were determined since these drugs respectively increase and decrease overall coronary blood flow (1 2)

MATERIALS AND METHODS

Details of the materials and methods used have been described elsewhere (3) These data on Rb^{86} uptake of different regions of the heart were obtained from the same dogs in which precordial monitoring was carried out.

Mongrel dogs weighing 6.7 to 14.3 Kg were anesthetized with sodium pentobarbital and infused intravenously with Rb^{86} at a continuously decreasing rate for periods of 5 to 90 minutes in order to attain nearly constant levels of Rb^{86} in the plasma of arterial blood. The radioactivity of whole blood was monitored in a small external arterio-venous shunt from which samples were taken at 5 minute intervals for determination of plasma Rb^{86} and potassium concentrations. Frequent recordings of heart rate and mean arterial blood pressure were made after 100 to 150 mg heparin had been given. Four dogs received a mean dosage of 2.5 μgm. per Kg per min. l-norepinephrine intravenously for 30 minutes and six dogs received a mean of 0.065 pressor units per Kg per min. Pitressin® for a similar period, during which time they also received Rb^{86} The dogs were sacrificed by rapidly opening the chest and removing the heart. Specimens were obtained from several regions of the heart, from the lung and liver and from the skeletal muscle of the pectoral region and spine.

Specimens obtained from the hearts of 29 dogs were as follows full thickness of the left ventricle in the apical region, full thickness of the left ventricle in the basilar region, full thickness of the mid-portion of the interventricular septum a portion of the thickest part of the left ventricle divided approximately into outer middle, and inner thirds a similar specimen from the right ventricle divided into inner and outer halves, and specimens of the full thickness of the right ventricle and from both auricles. The latter included the appendages and a portion of the adjacent wall of the auricle. The potassium and Rb^{86} concentrations of all specimens were determined after digestion in HNO_3.

METHODS OF ANALYSIS

The rationale of the methods of analysis and the details of the procedure have been presented elsewhere (3) It was assumed that Rb^{86} traced potassium in the myocardium that the individual portions of the myocardium could be considered homogeneous compartments

TABLE I

Mean potassium concentration of various regions of the hearts of 19 control dogs and the mean ratio of myocardial Rb^{86}/K to plasma Rb^{86}/K of the same regions of the hearts of eight dogs sacrificed more than 72 hours after Rb^{86} injection

Region of the myocardium	(Myocardial Rb^{86}/K ÷ Plasma Rb^{86}/K) × 100, at equilibrium	K conc. mEq per Kg
Mean of six specimens from the left ventricle	106 1 ± 7.6	82.2 ± 4 5
Outer third of left ventricle	108 0	83.3
Middle third of left ventricle	105 8	84 0
Inner third of left ventricle	104 0	80.3
Mean of three specimens from the right ventricle	103.3	81 6
Outer half of right ventricle	104.5	82.2
Inner half of right ventricle	102 9	80 9
Right atrium	115 6	59.5
Left atrium	114 1	67.2

[1] Supported by the R. A. Bilups Fund for Research in Heart Disease and aided by a *U S Public Health* Service Grant, H 143

6 These differences in the rate of Rb^{86} uptake are believed to be related to differences in the rate of effective blood flow, although other factors have not been eliminated

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5 The concentration of high density lipoproteins in obstructive jaundice may be predicted from knowledge of either the concentration of the cholesterol esters or the total or unesterified cholesterol concentration together with the per cent of cholesterol esterified

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NET POTASSIUM MOVEMENT BETWEEN RESTING MUSCLE AND PLASMA IN MAN IN THE BASAL STATE AND DURING THE NIGHT [1]

By REUBIN ANDRES GORDON CADER, PETER GOLDMAN AND KENNETH L. ZIERLER

(From the Departments of Environmental Medicine and Medicine The Johns Hopkins University and Hospital Baltimore Md.)

(Submitted for publication July 5 1956 accepted January 24 1957)

Net movement of potassium between cells and extracellular fluid has been demonstrated in a variety of experimental conditions by direct analysis of muscle by metabolic balance techniques and by measurement of differences in potassium concentration in arterial blood and venous blood draining muscle It has for example been demonstated that potassium leaves muscle during exercise, anoxia, potassium depletion, and under the influence of acidosis and of adrenal cortical hormones (1)

The present study demonstrates that there are, *in addition diurnal fluctuations in movement* of potassium between skeletal muscle and extracellular fluid Net movement of potassium between plasma and muscles of the forearm has been determined in human subjects by a technique which permits continuous monitoring of movement of potassium in relatively undisturbed muscle. Concentration of potassium is measured in arterial plasma and in venous plasma draining the deep forearm tissues. Plasma flow through the forearm is measured by the dye-dilution method (2) The product of flow F and the difference in arteriovenous concentration A V defines $\dot{Q}$ the net quantity of potassium entering or leaving the forearm tissues per unit time.

METHODS

In vivo studies. Twenty-one male subjects (16 medical students and laboratory personnel and five convalescent ambulatory patients) were studied in the basal state between the hours of 10 A.M. and 1 P.M. Three medical students, including two of the 21 subjects above, were *also studied throughout the night and morning hours, i.e.,* from 10 P M to 11 A.M Studies of carbohydrate metabolism in the first 13 of these subjects have been reported elsewhere (3 4) and subject numbers in those papers correspond to those reported here. All subjects had their last meals between 6 and 7 P M and were at rest for at least one hour before blood was sampled. The arm was supported comfortably and there was no overt activity of the forearm muscles during the period of study Blood from the hand was eliminated by inflating to above systolic pressure a cuff about the wrist, applied five minutes prior to blood sampling Blood was collected in heparinized syringes metabolic inhibitors were not used. Consecutive pairs of arteriovenous samples were obtained at 12 to 60 minute intervals. Processing of blood was as expeditious as possible. Immediate centrifugation of the blood samples was carried out for ten minutes, plasma was transferred and re-centrifuged twice. A few visibly hemolyzed samples were discarded. Since 0.05 per cent hemolysis can be detected visually the maximum increase in plasma potassium concentration resulting from undetected hemolysis was 0.02 mEq per L. Handling of samples for potassium analysis was carried out at room temperature (25 C) to minimize potassium and water shifts in erythrocytes (5) prior to their separation from plasma.

In vitro studies. A sample of antecubital venous blood was obtained anaerobically in a heparinized syringe. A drop of mercury was introduced and the sample was thoroughly mixed. Samples of blood were transferred anaerobically into duplicate syringes. Into one of these, 100 per cent O_2 was introduced, and into the other a mixture of 5.0 to 6.4 per cent CO_2 in N_2. Gas volumes were varied to obtain the desired range of gas concentrations in the blood samples. Syringes were capped and the blood and gas phases were equilibrated at room temperature by rotation for ten minutes. Gases were expelled and blood was then handled as in the *in vivo* studies Hematocrit determinations (Wintrobe) were performed in duplicate. Tubes were centrifuged for one hour at 1500 g and no correction was made for trapped plasma. In some studies, concentrations of O_2 and CO_2 were determined by the method of Van Slyke and Neill (6)

Potassium analysis. Duplicate or triplicate dilutions of plasma were made and concentrations were measured

[1] This work was performed under a contract between the Office of Naval Research, Department of the Navy and The Johns Hopkins University (NR 113-241) and was further supported by grants in aid from the National Institutes of Health, Department of Health, Education, and Welfare (A 750), and the Muscular Dystrophy Associations of America, Inc.

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In vivo studies. Twenty-one male subjects (16 medical students and laboratory personnel and five convalescent ambulatory patients) were studied in the basal state between the hours of 10 A.M. and 1 P.M. Three medical students, including two of the 21 subjects above, were also studied throughout the night and morning hours, i.e., from 10 P.M. to 11 A.M. Studies of carbohydrate metabolism in the first 13 of these subjects have been reported elsewhere (3, 4) and subject numbers in those papers correspond to those reported here. All subjects had their last meals between 6 and 7 P.M. and were at rest for at least one hour before blood was sampled. The arm was supported comfortably and there was no overt activity of the forearm muscles during the period of study. Blood from the hand was eliminated by inflating to above systolic pressure a cuff about the wrist, applied five minutes prior to blood sampling. Blood was collected in heparinized syringes; metabolic inhibitors were not used. Consecutive pairs of arteriovenous samples were obtained at 12 to 60 minute intervals. Processing of blood was as expeditious as possible. Immediate centrifugation of the blood samples was carried out for ten minutes, plasma was transferred and re-centrifuged twice. A few visibly hemolyzed samples were discarded. Since 0.05 per cent hemolysis can be detected visually, the maximum increase in plasma potassium concentration resulting from undetected hemolysis was 0.02 mEq. per L. Handling of samples for potassium analysis was carried out at room temperature (25° C) to minimize potassium and water shifts in erythrocytes (5) prior to their separation from plasma.

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with the Beckman Model DU flame photometer The precision of the analysis is as follows the standard error of the estimate of the mean concentration from duplicate analyses of a single blood sample is 0 04 mEq per L. The standard error of the estimate of a single arteriovenous difference then is 0 06 mEq per L. for samples diluted in duplicate. On the average, three pairs of arterial and venous samples were obtained from each subject in the basal state and only the mean of these multiple samples is reported. In general the standard error (analytical) of the estimate of *mean* A-V difference to be presented for each subject in Table I is 0 03 mEq per L. For the entire series of 21 subjects the standard error of the estimated overall mean A-V difference is less than 0 01 mEq per L.

RESULTS

Potassium movement in the basal state

Sixty-five individual A-V differences were obtained in 21 subjects studied 16 to 19 hours postprandially In only seven samples was the arterial concentration higher than the venous The mean A-V difference in 19 of the 21 subjects was negative, the grand mean being − 0 23 mEq per L (Table I) The net loss of potassium from tissue to blood in 15 subjects averaged 0 66 μEq per min per 100 ml forearm tissue Q can be calculated in terms of forearm *muscle* by multiplying 0 66 by 4/3 (see Reference 3), giving a mean loss of 0 88 μEq per min per 100 g forearm muscle

TABLE I

Movement of potassium in the basal state *

Subject	Number of pairs	A_K *mEq/L.*	$(A-V)_K$ *mEq/L.*	$\dot{Q}_K$ *μEq/min/100 ml forearm*
1	6	4 36	−0 31	−0 88
2	6	4 19	−0 46	
3	6	4 07	−0 15	
4	3	3 90	−0 23	−0 50
5	3	4 28	−0 01	
6	3	3 90	−0 23	−0 37
7	2	3 77	−0 16	
8	1	3 82	−0 14	−0 17
9	1	3 72	−0 03	−0 10
10	1	4 49	−0 38	−0 83
11	3	4 51	−0 10	
12 (C)	2	3 91	−0 07	−0 18
13 (K)	3	4 04	−0 09	−0 17
14	3	4 14	−0 96	−2 19
15	3	3 89	−0 44	−1 86
16	3	4 03	+0 01	+0 11
17	3	3 98	−0 64	−2 69
18	4	3 94	−0 28	
19	3	3 65	+0 23	+0 54
20	3	4 04	−0 16	−0 21
21	3	3 95	−0 19	−0 43
Mean	3	4 03	−0 229	−0 662
S D		0 233	0 253	0 902
S E M		0 051	0 055	0 233

* A_K is the concentration of potassium in arterial plasma $(A-V)_K$ is the arteriovenous difference in plasma concentration of potassium $\dot{Q}_K$ is the calculated net potassium movement, the minus sign indicates that net movement was from muscle to plasma Values in each column are the means for each subject Plasma flow was not successfully measured in six subjects (see Reference 2), and $\dot{Q}_K$ for each of these was therefore not calculated S D is the standard deviation and S E M is the standard error of the mean

TABLE II

Movement of potassium during the night *

Sample	Time	A_K *mEq/L.*	$(A-V)_K$ *mEq/L*	$\dot{Q}_K$ *μEq/min/100 ml forearm*
Subject C				
1	11 43 P M	3 91	−0 32	−0 89
2	1·00 A M	3 91	+0 03	+0 08
3	2·00	4 17	+0 35	+1 12
4	3·00	3 86	−0 09	−0 25
5	5·00	3 81	−0 09	−0 40
6	7·00	3 88	−0 04	−0 07
7	9·00	3 84	−0 17	−0 42
8	10·00	3 90	−0 03	−0 13
9	11·00	3 92	−0 11	−0 23
Subject K				
1	11.53 P M	3 93	−0 12	−0 23
2	1·00 A M	3 95		
3	2·00	3 94	+0 01	+0 02
4	3 30	3 92	−0 02	−0 04
5	5·00	3 89	−0 11	−0 17
6	6.30	4 16	−0 06	−0 07
7	8·00	4 17	+0 02	+0 04
8	10 13	4 03	−0 09	−0 18
9	10 35	4 00	−0 09	−0 17
10	11·02	4 10	−0 09	−0 17
Subject N				
1	10·00 P M	3 86	−0 31	
2	11.30	3 84	+0 10	
3	1·00 A M	3 76	+0 04	
4	3 00	3 82	+0 05	
5	5·00	3 90	+0 04	
6	7·00	3 86	+0 02	
7	8·00	3 82	+0 01	
8	9·00	3 90	+0 12	

* See legend to Table I for explanation of symbols

Potassium movement during the night

Results on the three subjects are given in Table II and Figure 1 Measurement of plasma flow was made in subjects C and K but was unsuccessful in subject N (2) It appears that between the hours of 1 and 8 A M there is no net movement of potassium, the overall mean and median A-V difference during this time being + 0 01 mEq per

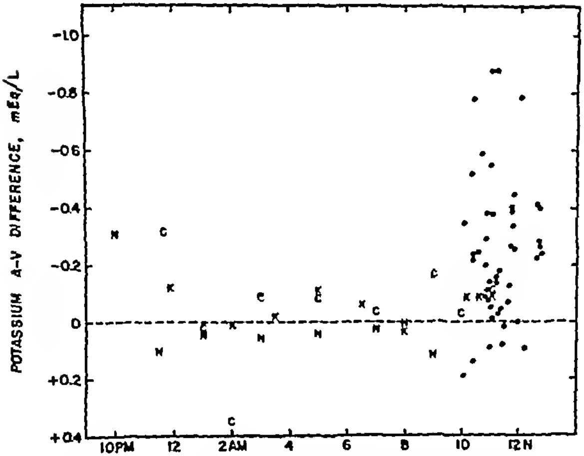

FIG. 1 TIME COURSE OF ARTERIOVENOUS DIFFERENCE IN PLASMA POTASSIUM CONCENTRATION

C, K and N refer to subjects followed during the night. Black dots represent all A V differences obtained in nineteen additional subjects studied only between 10 A.M. and 1 P.M

L., and only one of the 15 A V differences lies outside the range expected from analytical error alone These results are in contrast to the larger series of results obtained during the late morning hours In the few observations made prior to 1 A.M there seems also to be real loss of potassium from the forearm

In summary the general trend is one of potassium loss from muscle prior to 1 A M little or no net movement during most of the night then increasing potassium loss in the later morning hours.

Evidence that the observed A V differences in potassium concentration represent net movement of potassium between forearm muscle and blood

1) Transiently negative A-V differences might be observed if the concentration of potassium in arterial plasma were falling during the period of study with consequent net diffusion of potassium from interstitial fluid into capillaries However only very minor fluctuations in concentration of arterial potassium occurred and there was no consistent directional trend On the average the concentration decreased 0 004 mEq per L. ± 0 018 (S.E.M) in consecutive samples

2) Since potassium escapes from anoxic muscle (7), it was necessary to demonstrate that no leak of blood from ischemic hand tissues distal to the pressure cuff at the wrist occurred Evidence that there was no such leak has been previously presented (3)

3) The chief source of the potassium added to venous blood might be forearm tissues other than muscle that is skin tendon or bone The placement of the venous catheter deeply into the forearm muscle mass minimizes possible contribution from skin Of the potassium in the remaining tissues there is probably 20 times as much in muscle as there is in bone and tendon combined if concentrations in human tissues are similar to those in the rat (8) Nevertheless a portion of the observed potassium loss may be from tissues other than muscle

4) *In vitro* studies of net potassium and water movement between blood cells and plasma were made to examine the possibility of significant shift of potassium of water or of both either (a) in the arm as arterial blood becomes venous or (b) in shed blood during the several minutes which elapse before cells are separated from plasma.

with the Beckman Model DU flame photometer The precision of the analysis is as follows the standard error of the estimate of the mean concentration from duplicate analyses of a single blood sample is 0 04 mEq per L. The standard error of the estimate of a single arteriovenous difference then is 0 06 mEq per L. for samples diluted in duplicate. On the average, three pairs of arterial and venous samples were obtained from each subject in the basal state and only the mean of these multiple samples is reported. In general the standard error (analytical) of the estimate of *mean* A-V difference to be presented for each subject in Table I is 0 03 mEq per L. For the entire series of 21 subjects, the standard error of the estimated overall mean A-V difference is less than 0 01 mEq per L.

RESULTS

Potassium movement in the basal state

Sixty-five individual A-V differences were obtained in 21 subjects studied 16 to 19 hours postprandially In only seven samples was the arterial concentration higher than the venous The mean A-V difference in 19 of the 21 subjects was negative, the grand mean being − 0 23 mEq per L (Table I) The net loss of potassium from tissue to blood in 15 subjects averaged 0 66 μEq per min per 100 ml forearm tissue Q can be calculated in terms of forearm *muscle* by multiplying 0 66 by 4/3 (see Reference 3), giving a mean loss of 0 88 μEq per min per 100 g forearm muscle

TABLE I

Movement of potassium in the basal state *

Subject	Number of pairs	A_K mEq/L.	$(A-V)_K$ mEq/L.	$\dot{Q}_K$ μEq/min/100 ml forearm
1	6	4 36	−0 31	−0 88
2	6	4 19	−0 46	
3	6	4 07	−0 15	
4	3	3 90	−0 23	−0 50
5	3	4 28	−0 01	
6	3	3 90	−0 23	−0 37
7	2	3 77	−0 16	
8	1	3 82	−0 14	−0 17
9	1	3 72	−0 03	−0 10
10	1	4 49	−0 38	−0 83
11	3	4 51	−0 10	
12 (C)	2	3 91	−0 07	−0 18
13 (K)	3	4 04	−0 09	−0 17
14	3	4 14	−0 96	−2 19
15	3	3 89	−0 44	−1 86
16	3	4 03	+0 01	+0 11
17	3	3 98	−0 64	−2 69
18	4	3 94	−0 28	
19	3	3 65	+0 23	+0 54
20	3	4 04	−0 16	−0 21
21	3	3 95	−0 19	−0 43
Mean	3	4 03	−0 229	−0 662
S D		0 233	0 253	0 902
S E M		0 051	0 055	0 233

* A_K is the concentration of potassium in arterial plasma $(A-V)_K$ is the arteriovenous difference in plasma concentration of potassium Q_K is the calculated net potassium movement the minus sign indicates that net movement was from muscle to plasma Values in each column are the means for each subject Plasma flow was not successfully measured in six subjects (see Reference 2), and $\dot{Q}_K$ for each of these was therefore not calculated S D is the standard deviation and S E M is the standard error of the mean

TABLE II

Movement of potassium during the night *

Sample	Time	A_K mEq/L	$(A-V)_K$ mEq/L.	$\dot{Q}_K$ μEq/min/100 ml forearm
Subject C				
1	11 43 P M	3 91	−0 32	−0 89
2	1·00 A M	3 91	+0 03	+0 08
3	2·00	4 17	+0 35	+1 12
4	3·00	3 86	−0 09	−0 25
5	5·00	3 81	−0 09	−0 40
6	7·00	3 88	−0 04	−0 07
7	9·00	3 84	−0 17	−0 42
8	10·00	3 90	−0 03	−0 13
9	11·00	3 92	−0 11	−0 23
Subject K				
1	11 53 P M	3 93	−0 12	−0 23
2	1·00 A M	3 95		
3	2 00	3 94	+0 01	+0 02
4	3 30	3 92	−0 02	−0 04
5	5·00	3 89	−0 11	−0 17
6	6 30	4 16	−0 06	−0 07
7	8·00	4 17	+0 02	+0 04
8	10 13	4 03	−0 09	−0 18
9	10 35	4 00	−0 09	−0 17
10	11·02	4 10	−0 09	−0 17
Subject N				
1	10·00 P M	3 86	−0 31	
2	11 30	3 84	+0 10	
3	1·00 A M	3 76	+0 04	
4	3·00	3 82	+0 05	
5	5·00	3 90	+0 04	
6	7·00	3 86	+0 02	
7	8·00	3 82	+0 01	
8	9·00	3 90	+0 12	

* See legend to Table I for explanation of symbols

Potassium movement during the night

Results on the three subjects are given in Table II and Figure 1 Measurement of plasma flow was made in subjects C and K but was unsuccessful in subject N (2) It appears that between the hours of 1 and 8 A M there is no net movement of potassium, the overall mean and median A-V difference during this time being + 0 01 mEq per

potassium shift in the *in vitro* studies both quantitatively very small

Venous plasma concentrations, as measured in the forearm experiments might be corrected for possible movements of water and potassium between erythrocytes and plasma by subtracting 0 03 mEq per L However in view of the fact that a water shift has been shown not to occur in the *in vivo* experiments, there is some justification for not correcting for that portion (60 per cent) of the *in vitro* potassium A-V difference which can be attributed to a water shift. The correction then would be only 0 01 mEq per L., a value so small that it was deemed inadvisable to carry out any correction

From these *in vitro* experiments it cannot be stated whether these erythrocyte-plasma exchanges occurred during passage of blood through the arm or during the handling of the shed blood

DISCUSSION

These studies demonstrate that, in addition to factors described previously as influencing transcellular movement of potassium there is a net loss of potassium from skeletal muscle to extracellular fluid in the late morning hours During the night there appears to be no net movement, *so that in the 12 hours of the 24 included in this* study there is an overall loss of potassium from muscle Restitution has not been demonstrated it is likely that this occurs with potassium ingestion in food

The morning loss of potassium from muscle averages 0 88 μEq per min per 100 g muscle, or 0.5 per cent of the intracellular potassium per hour If extrapolation from forearm to total body muscle is valid, an increase in the extracellular concentration of potassium of 1 0 mEq per L. would be expected in one hour Yet, arterial concentration remains constant It is clear that potassium must leave the extracellular fluid at about the rate at which it is added to extracellular fluid from muscle. Studies by others (10 11) of the diurnal variation of urinary excretion of potassium show a striking similarity to the pattern of net potassium movement from muscle. That is excretion falls to minimal levels during the night, and rises sharply to a maximum output at 10 or 11 A.M. (compare Figure 1) It would appear that urinary loss is the likely fate of the bulk of the potassium added to extracellular fluid from muscle,

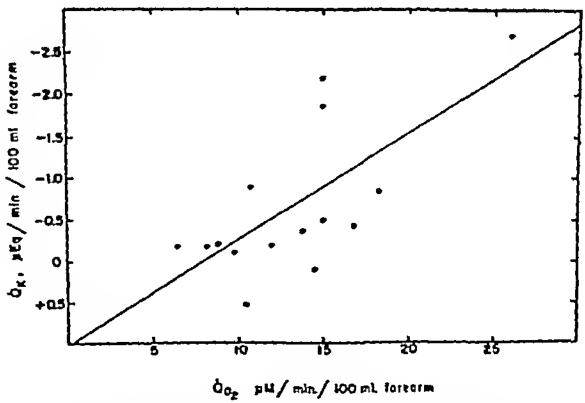

FIG. 2. CORRELATION BETWEEN OXYGEN CONSUMPTION OF FOREARM TISSUES AND MOVEMENT OF POTASSIUM BETWEEN FOREARM TISSUES AND BLOOD

Each dot represents the mean value for each subject studied in the basal state. Line of regression calculated by "least squares" method. Probability of correlation by chance, < 0 01

In case (a), these shifts to a new equilibrium state might be considered as accompaniments of the classical "chloride shift" (9). That is, either movement of water into erythrocytes or potassium out of erythrocytes, or both, might lead to a higher venous than arterial plasma concentration of potassium.

In case (b) it is conceivable that, as shed arterial and venous blood cool from 38°C toward room temperature of 25°, net potassium uptake by arterial erythrocytes might occur at a faster rate than by venous erythrocytes, or, net water uptake by arterial erythrocytes might occur at a slower rate than by venous erythrocytes.

These possible artefacts could be avoided by determining potassium concentrations in whole blood rather than in plasma. Concentrations in whole blood are so high, however, that a small analytical error percentagewise would become intolerably large in detecting small net potassium shifts.

In both cases (a) and (b), in order for water shifts to account for the observed differences in potassium concentration, the volume of packed cells in venous blood would have to exceed that in arterial blood by 2.3 ml. per 100 ml. blood on the average. In 35 pairs of arterial and venous blood samples obtained in 12 of the subjects, there was no significant A-V difference, the mean being only +0.2 ml. per 100 ml. ± 0.2 (S.E.M.). Water shifts, then, are not responsible for the observed potassium differences. However, since the possibility of a potassium shift in erythrocytes remained, *in vitro* studies were devised so that the changes in gas content occurring in blood flowing through the arm would be reversed. The sample of venous blood equilibrated with oxygen becomes "arterialized": O_2 is gained and CO_2 is lost. The paired sample of venous blood which is exposed to the $CO_2 - N_2$ mixture serves as a control: the blood is subjected to the same manipulative procedures but remains venous.

In eleven *in vitro* experiments (Table III) there is a significantly greater concentration of potassium in the "venous" than in the "arterial" sample, but the mean difference, 0.03 mEq. per L., is only 1/7 the mean difference in the experiment upon the forearm. The difference between *in vivo* and *in vitro* A-V differences is highly significant ($p < 0.01$). However, unlike the forearm experiments, a significant difference in "arterial" and "venous" hematocrits occurred in the *in vitro* studies. In only one of ten pairs was this water shift large enough to account completely for the observed difference in potassium concentration (Table III, columns headed "% Water shift" and "% Change in potassium"). There therefore appears to be not only a water shift but also a direct

TABLE III

*Movement of potassium between red cells and plasma with changes in O_2 and CO_2 contents**

Expt.	A_K	$(A-V)_K$	$(A-V)_{O_2}$	$(A-V)_{CO_2}$	$(A-V)_{Hct}$	% Water shift	% Change in potassium
A	3.67	−0.01	9.4	−1.6			−0.2
B	3.81	−0.13	13.9	−1.6	−0.4	−0.7	−3.4
C	3.95	−0.01	15.4	−3.4	−1.4	−2.6	−0.4
D	3.68	+0.01			−0.1	−0.2	+0.3
E	3.74	−0.03	13.4	−2.3	−0.2	−0.4	−0.7
F	3.73	−0.05			−0.3	−0.6	−1.4
G	3.91	0			+0.1	+0.3	0
H	4.19	−0.04			0	0	−1.0
I	3.61	−0.04			−0.2	−0.3	−1.3
J	4.05	−0.01	10.8	−5.6	0	0	0
K	4.01	−0.07	11.6	−6.3	−0.7	−1.2	−1.7
Mean	3.850	−0.034	12.4	−3.5	−0.31	−0.57	−0.96†
S.E.M.		0.0120			0.140	0.260	0.338

* A refers to the concentration in the "arterialized" sample; V, to that in the venous sample; A-V, to the arteriovenous difference. Potassium concentration is in mEq. per L. plasma; gas concentrations are in ml. per 100 ml. blood. Hematocrit, Hct, is in ml. packed cells per 100 ml. blood. % Water shift $= \frac{(V-A)_{plasmacrit}}{A_{plasmacrit}} 100$. % Change in potassium $= \frac{(A-V)_K}{A_K} 100$.

† Experiment A omitted from calculation of the mean since per cent water shift was not measured in this subject.

fluid (and therefore, of potassium) with glycogen deposition With such a low uptake of glucose it would not be expected that either of these two processes occurred in fact breakdown of previously deposited glycogen would seem more likely and this might lead to a loss of associated potassium from muscle. Available data in the literature do not permit, however an acceptable estimate of the quantity of glycogen breakdown required to acconnt for the observed potassium movement. The mean R Q of the forearm in these subjects (0.74 ± 0.02) suggests that glucose oxidation and therefore glycogenolysis must be minor and there is other evidence suggesting that little breakdown of glycogen in muscle is to be expected (3) There was also no correlation between movement of potassium and forearm R.Q

Carbon dioxide It has been demonstrated that acute acidosis both respiratory and metabolic, leads to loss of potassium from muscle (12 13) In the forearm experiments there was a highly significant correlation between CO_2 production by the forearm and net potassium movement (Figure 3) Greater CO_2 production does not, however necessarily imply increased intracellular acidosis since intracellular CO_2 concentration is a function not only of the rate of CO_2 production but also of the rate of CO_2 removal CO_2 concentration in venous blood or the A V difference in CO_2 concentration might then be better indices of tissue pH, neither of these correlated with the net movement of potassium. Since neither pH nor pCO_2 was measured it is not possible from these experiments to be certain what role tissue pH might play

In the three subjects studied during the night there was no correlation between movement of potassium and of any other metabolite.

SUMMARY

There is net movement of potassium out of resting forearm muscle into plasma in the late morning hours in fasted subjects From 1 A M to 10 A.M no net movement occurs From 10 A.M to 1 P M the mean A V difference in 21 subjects was − 0.23 mEq per L. The net loss in 15 subjects averaged 0.88 μEq per min per 100 g muscle or about 0.5 per cent per hour of intracellular potassium

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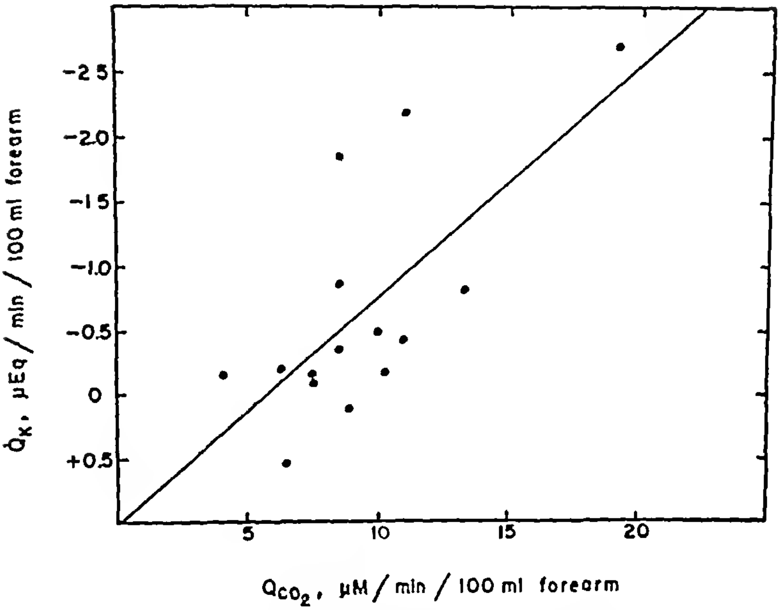

FIG 3 CORRELATION BETWEEN CO_2 PRODUCTION BY FOREARM TISSUES AND MOVEMENT OF POTASSIUM BETWEEN FOREARM TISSUES AND BLOOD

Each dot represents the mean value for each subject studied in the basal state. Line of regression calculated by "least squares" method. Probability of correlation by chance, < 0 01

some potassium may, however, enter the cells of other organs, for example, liver

The similarity of the time course of movement of potassium out of muscle and into urine suggests that the two are causally related It is not known which of these processes may be primary, or indeed whether both are secondary to another common mechanism The association of adrenocortical activity with electrolyte metabolism and the striking diurnal variation of adrenocortical activity (11) suggest that this may be the common trigger to muscle and kidney Others (10) have suggested that the diurnal rhythm of potassium excretion is caused by cyclic changes in renal tubular metabolism with consequent changes in intracellular pH These two suggestions are not mutually exclusive

Carbohydrate metabolism of the forearm was studied in these subjects by measuring the net movement between forearm tissues and blood of glucose, lactate, oxygen and carbon dioxide simultaneously with potassium on the same samples of blood Data on carbohydrate metabolism in the first 13 of these subjects have been reported previously (3, 4) The relation between movement of potassium and of these metabolites in the basal state was examined

Oxygen Since anoxia of muscle leads to loss of potassium, it might have been expected that subjects with the lowest O_2 consumption would have the greatest loss of potassium from muscle However, the reverse was true (Figure 2)

Lactate Lactate was produced by the resting forearm (mean = 0 44 μM per min per 100 ml forearm $\pm$ 0 11 S E M) It is possible that there are small areas of muscle which are intermittently ischemic, perhaps as a result of rhythmic vasomotor activity, which accounts for this anaerobic metabolism If so, correlation between lactate production by muscle and potassium loss from muscle would be expected There was, however, no correlation

Glucose Glucose uptake by forearm tissue was small, averaging only 0 50 $\pm$ 0 17 μM per min per 100 ml forearm, and no correlation with potassium movement was present The movement of potassium into cells along with glucose is generally attributed either to a) an increase in concentration within the cell of the potassium salt of the hexose phosphates or b) an increase of intracellular

MOVEMENT OF POTASSIUM INTO SKELETAL MUSCLE DURING SPONTANEOUS ATTACK IN FAMILY PERIODIC PARALYSIS [1]

By KENNETH L. ZIERLER AND REUBIN ANDRES

(From the Departments of Environmental Medicine and Medicine, The Johns Hopkins University and Hospital, Baltimore, Md.)

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It has been appreciated since the classical studies of Biemond and Daniels (1) and Aitken, Allott, Castleden, and Walker (2) that the concentration of potassium in serum falls during attacks in patients with typical family periodic paralysis. Allott and McArdle (3), Pudenz, McIntosh, and McEachern (4) and Ferrebee, Atchley, and Loeb (5) showed that the quantity of potassium excreted in urine was reduced during attacks of paralysis. These observations implied that during attacks potassium shifted from extracellular to some intracellular space. Metabolic balance studies by Danowski, Elkinton, Burrows, and Winkler (6) confirmed this hypothesis. The question of which intracellular space accepted the potassium that shifted during attacks remained unanswered.

Recently it has been reported from this laboratory that there is a diurnal variation in normal man in exchange of potassium between skeletal muscle and extracellular water (7). During the hours between 10 P.M. and 12 noon, that is, 4 to 18 hours after the last meal, with the subject at rest, potassium moves from muscle to blood, the rate declining to a minimum or even reversing slightly (potassium moves from blood to muscle) between the hours of midnight and 7 A.M., and then rising again to a peak between 10 A.M. and noon. It is characteristic of family periodic paralysis that most attacks begin during the middle of the night (8). This suggested that in patients with this disease there may be an exaggeration of the diurnal variation in potassium movement, and that movement of potassium from extracellular fluid into skeletal muscle during the night might be responsible for the attacks. It is the purpose of this report to present data indicating that this is the case.

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METHODS

Two subjects were studied. Both had well-documented family periodic paralysis with a history of spontaneous attacks of flaccid paralysis frequently having their onset in the middle of the night and associated with definite hypokalemia.

Subject M. H., a 14-year-old boy weighing 57 Kg., had had episodes of weakness and paralysis since age 12. All the attacks began between 11 P.M. and 9 A.M. They were relieved or prevented by administration of potassium chloride, and serum potassium concentration during at least one attack was found to be low. The severity of his attacks was much less than that of the second patient.

Subject M. H. was studied on three occasions.

Study I was performed between 11:30 A.M. and 12 noon during spontaneous recovery from a mild attack which had begun spontaneously during the night. He had had no food since supper at 6 P.M. the evening prior to study.

Study II covered the hours between midnight and 7 A.M. The last meal was at 6 P.M. When a spontaneous attack did not occur by 2:20 A.M., an attack was induced by administration of glucose and of insulin.

Study III covered the hours between 1 A.M. and 7 A.M. During the afternoon prior to study M. H. exercised heavily on a treadmill and at 6 P.M. ate a large dinner. There was no clinical evidence of an attack during the time of this study.

Subject A. B., a 24 year-old college student weighing 77 Kg., was a member of a family of whom six members had clinical evidence of periodic paralysis. His attacks began at age 12. There were one to three severe attacks and more frequent mild attacks each month until age 18 when he was first placed on prophylactic potassium therapy. Circumstances under which attacks occurred were quite predictable. Attacks followed heavy exercise and high carbohydrate meals, were aborted by mild exercise and ingestion of potassium chloride. Onset of attacks was usually in the middle of the night and some of his attacks were quite severe, involving not only the extremities but also respiratory muscles.

Subject A. B. was studied on one occasion. Prophylactic potassium therapy was discontinued 24 hours prior

TABLE I

*Net movement of potassium during spontaneous nocturnal attack—Subject A B**

Time A.M	A mEq./L.	A V mEq./L.	Q mEq./min./100 ml forearm	Oral KCl g	Muscle strength	Tendon reflexes†
12 12	2 47	0 74	1.8		Normal	B-1 T 2 K 2 2
1:08	2 11	0 60	1.3			
1 25 to 1 55					Rapid decrease in arms legs fairly strong	B-0 T-0 K 1 0 A 2 1
2·08	2 11	0 55	0 9			
2.55					Maximal inspiration reduced but no dyspnea. Neck flexion very weak.	
3:08	1 91	0.44	0 8			
4:00	1.85	0 49	1 1		Extremities almost totally paralyzed (Severe EKG abnormalities)	
4.10				5		
4·40				5	Subjective improvement. No objective change.	B-0 T 1 K-0 0 A 2 1
5·48	2 75	0 61	0 8		EKG less abnormal	
6.25	2.73	0.36	0 7	5	Extremity strength slightly improved. Inspiration stronger (EKG—return to severe changes.)	B-0 T 2 K 1 0 A 2 1
7 10				5		
7 13	3 11	−0 20	−0 4		Head can be raised off pillow	
7·43	3 45	−0.50	−0 8			
9:00	5 40	−0 61	−1 3		Nearly recovered	B-2 T 1 K-3 2 A 2 2
10·03	5 91	−0.33	−0 7		Essentially normal	

* A = concentration of potassium in arterial plasma A V = arteriovenous difference in plasma concentration of potassium Q = net uptake (or net release when value is negative) of potassium by forearm tissues.

† B = biceps, T = triceps K = knee, A = ankle Only the right biceps and triceps reflexes could be tested metabolic studies were made on the left arm 0 = absent 1 = reduced 2 = normal, 3 = hyperactive. In the knee and ankle jerks, the first number refers to the right side the second to the left side.

to this study He received 100 g of glucose at 3.30 P.M ate his usual supper at 5.30 P.M with extra dessert plus 100 g of glucose at 6.30 P.M. Measurements were made between midnight and 10 A.M A severe attack occurred and it was necessary to treat him with KCl.

Measurements were made of the metabolism of forearm muscles with the subject at rest. Uptake and release of potassium, O_2, CO_2, glucose and lactate were calculated as described previously (7 9) as the products of forearm blood flow and arteriovenous differences of the metabolites. Venous blood was obtained through a catheter placed in a deep vein draining forearm muscles. Arterial blood was from the brachial artery A pressure cuff about the wrist, inflated to greater than systolic pressure, excluded blood flow to hand and wrist during periods of blood collection.

Certain extrapolations of the data are made. In these it is assumed that uptake or output per min. per 100 ml. of forearm can be converted to uptake or output per min. per 100 g of forearm muscle by multiplying by ⅚, a conversion factor estimated previously (9) It is further assumed that extracellular fluid weighs 20 per cent of body weight and that total muscle mass is 40 per cent of body weight.

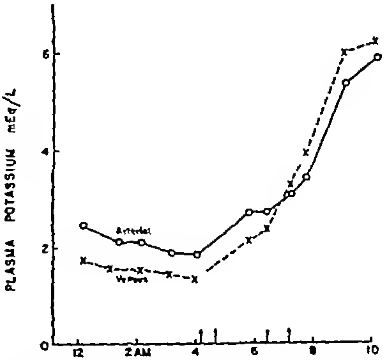

FIG. 1 ARTERIAL AND VENOUS PLASMA POTASSIUM CONCENTRATIONS DURING ATTACK AND RECOVERY IN PATIENT A B

At each time indicated by arrows 5 g KCl was given orally

RESULTS AND DISCUSSION

Potassium Movement During Spontaneous Attack and Spontaneous Recovery

Subject A B (Table I and Figures 1 and 2)

Arterial potassium concentration was already reduced greatly by the time the first sample was drawn at midnight and continued to fall for the next four hours, after which it was necessary to treat the patient by administration of KCl In subject A B, between midnight and 4 A M, extraction of potassium (*i e*, A-V difference divided by arterial concentration) was approximately 25 per cent. In normal subjects little or no extraction of potassium occurs during these hours

During the attack potassium moved from plasma into muscle at a rate of 1.2 μEq per min per 100 ml forearm Between midnight and 4 A M, concentration of potassium in arterial plasma fell 0 6 mEq per L, a loss of about 9 5 mEq of potassium from total extracellular fluid During that time about 2 mEq of potassium moved from plasma into muscles of one forearm If all skeletal muscles were behaving identically, about 110 mEq of potassium might have moved from extracellular fluid into muscle in the four-hour period Since this figure is about 12 times as large as the estimated loss of extracellular potassium it follows either that forearm muscles were extracting potassium from plasma at a rate about 12 times greater than the average muscle or that potassium was added to plasma from some nonmuscular source

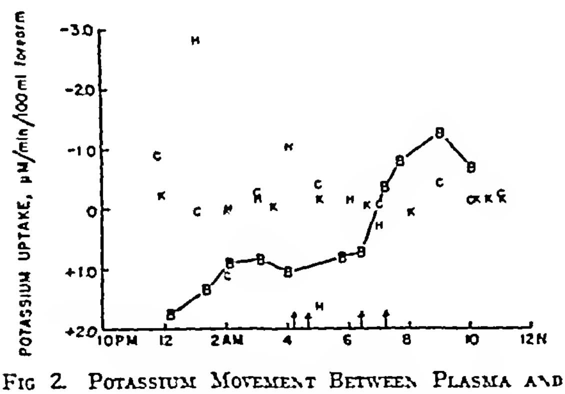

FIG 2. POTASSIUM MOVEMENT BETWEEN PLASMA AND MUSCLE DURING THE NIGHT

Positive values are uptake, negative values are release from muscle. B subject A. B during attack and recovery, K and C, normal subjects, H, subject M H who had periodic paralysis but failed to get an attack during this study Arrows indicate oral administration of 5 g KCl to A. B

Although the mass of potassium lost from extracellular fluid was large from the viewpoint of extracellular potassium, if it all went into skeletal muscle this quantity of potassium would be sufficient to raise the average concentration of intramuscular potassium by only a few per cent, an amount too small to be detected by available methods

When KCl was administered, although there was some elevation of arterial potassium concentration, extraction of potassium remained constant for the next two and one-half hours and potassium continued to move into muscle until the direction of movement was reversed at 7 A M, several hours earlier than the onset of accelerated output of potassium by muscle in normal subjects

In summary, an attack of paralysis developed in the middle of the night about seven hours after a heavy carbohydrate meal Potassium moved from plasma into skeletal muscle, concentration of potassium in plasma was reduced and paralysis occurred Efforts to terminate the attack by oral administration of potassium failed to do so during a two-hour period in which potassium continued to move from plasma into muscle and there was only a small increase in concentration of arterial potassium Presumably, during these two hours potassium movement out of extracellular space into muscle almost kept pace with intestinal absorption of administered potassium Later in the morning the direction of potassium movement reversed, potassium moved out of muscle into extracellular space at a rate greater than that seen in normal subjects during these hours Concentration of potassium in arterial plasma rose abruptly and the attack ended Although the rise in concentration of potassium in arterial plasma may have been the result of absorption of administered potassium from the gut as well as of outpouring of potassium from skeletal muscle, the latter factor alone was sufficient to account for the observed rise in plasma concentration

Subject M H, Study I (Table II, Figure 3)

Subject M H failed to have a spontaneous attack during the time measurements of forearm

TABLE II

*Potassium release during spontaneous recovery from spontaneous attack—Subject M H **

Time	A	A-V	$\dot{Q}$
11.39	4 09	−1 05	−3 85
11.50	4.10	−1 15	−6.23
12·00	4.08	−0.87	−3 70

* Time is A.M Symbols as in Table I

metabolism were made. However, forearm metabolism was measured on the morning following a mild spontaneous attack from which he was recovering spontaneously At the time of the study there was no longer any gross weakness Deep tendon reflexes in the arms were normal but patellar and ankle responses were still slightly depressed Release of potassium from muscle was about six times greater than the average rate found in normal subjects during the same time of the day Presumably at some earlier hour during the attack there was a reduction in plasma potassium concentration. The rate at which potassium moved from muscle to extracellular fluid during recovery 3 to 5 mEq per hr per Kg of muscle was adequate to account for an increase in plasma potassium concentration. Despite the large contribution of potassium to venous plasma from muscle concentration of potassium in arterial plasma was stable at about 4 mEq per L during the period of observation This stability of arterial potassium could obtain only if potassium left the blood stream by some other route as fast as it entered from skeletal muscle.

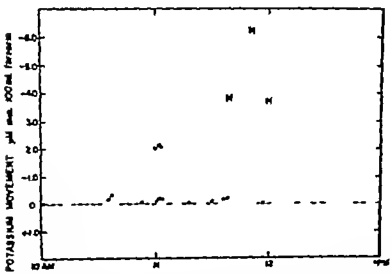

FIG. 3 POTASSIUM OUTPUT FROM MUSCLE TO PLASMA DURING SPONTANEOUS RECOVERY FROM A SPONTANEOUS ATTACK OF PARALYSIS IN SUBJECT M H.

● data from normal subjects in the basal state.

Potassium Movement During an Attack Produced by Administration of Glucose and Insulin

Subject M H Study II (Table III)

During Study II of subject M H., when a spontaneous attack had not occurred by 2.20 A.M a mild attack was produced by administration of glucose and insulin At 6.25 A M he was given 9 g KCl by mouth. Within 25 minutes there was some return of deep tendon reflexes

When KCl was administered there was a rapid rise in arterial potassium concentration despite a large increase in rate of potassium uptake by muscle. An uptake of this magnitude did not occur following potassium administration to subject A B at approximately the same time of day The difference in response is probably attributable to the effect of insulin since the behavior of blood glucose concentrations indicated that a potent insulin action occurred at the time of massive uptake of potassium

From the rise in concentration of potassium in

TABLE III

*Net movement of potassium during attack induced by glucose and insulin—Subject M H Study II **

Time A.M.	A mEq/L.	A-V mEq/L.	$\dot{Q}$ mEq/min/100 ml forearm	Comments
12.20	4 41	−0 06	−0 4	Strength and reflexes normal
1:20	4 44	−0 11	−0 8	
2.21	4.31	−0 16	−1.2	No symptoms or signs of impending attack.
2.25 to 2 40				130 g glucose in 240 ml. orange juice.
2·40				Insulin 20 U., subcutaneous.
3·02	4 09	−0.34	−2.6	
3.55	4 18	−0 01	−0 1	No change in strength or reflexes.
3.55 to 4·05				25 g glucose I V
4·07				Insulin 6 U., I V
4 41	3 68	0 08	0 7	Strength greatly diminished. Tendon reflexes absent.
5.29	3 73	0 26	1 8	
6·03	3 68	0 03	0.3	
6 15				Further weakness. Reflexes remain absent.
6.25				9 g KCl orally
6·40	4 95	0.68	6.6	Reflexes and strength returning
6.58	5.56	0 45	6 7	Almost complete recovery

* Symbols as in Table I

arterial plasma, from the measured uptake of potassium by muscles of the forearm and from reasonable assumptions of the size of extracellular space and total muscle mass in M H it can be estimated that of the 120 mEq of potassium administered about 20 mEq went into extracellular space and about 65 mEq went into skeletal muscle in 33 minutes, leaving about one-fourth unaccounted for Similar calculations made for the case of A B following the first 10 g of administered KCl (Table I) indicated that of the 135 mEq of potassium administered about 14 mEq went into extracellular space and about 35 mEq went into muscle in two hours, leaving about two-thirds of the potassium unaccounted for This suggests that the failure of oral administration of KCl to produce prompt restoration of normal potassium concentration in arterial plasma and clinical recovery may have been in part due to delayed absorption of potassium from the gut during the more severe attack suffered by A B

Normal Pattern of Potassium Movement During a Night When No Paralytic Attack Occurred

Subject M H, Study III (Figure 2)

During the early part of Study II, between midnight and 2 20 A M, subject M H had no spontaneous attack and potassium movement at this time was indistinguishable from that observed in normal men In Study III, between 1 A M and 7 A M, subject M H again had no spontaneous attack and potassium movement appeared to be normal

Fragmentary Observations on Other Abnormalities of Muscle Metabolism Occurring During a Spontaneous Attack of Paralysis (Table IV)

Certain observations discovered during the attack in A B which differ from those obtained in normal subjects are reported These differences may prove ultimately to be of no significance since the number of normal subjects studied is small On the other hand, even if they prove to be real departures from the normal it is not clear whether these differences are fundamental to the disease, whether they represent a defect parallel to but unrelated to the anomaly of potassium movement, whether they are in some way causally related to

TABLE IV

Blood flow, gas exchange and carbohydrate metabolism in muscle during spontaneous paralysis—Subject A B *

Time	Blood flow	Oxygen			Carbon dioxide			R Q	Glucose			Lactate			L/G	(G L)/O$_2$
		A	A V	Q	A	V A	Q		A	A V	Q	A	A V	Q		
12 12	4 10	8 62	2 69	11 03	21 61	2 48	10 17	0 92	6 42	0 98	4 02	0 595	−0 139	−0 570	7	203
1 08	3 81	8 93	2 03	7 73	21 28	2 99	11 39	1 47	6 47	0 56	2 13	0 655	0 071	0 271	None	176
2 03	2 78	8 81	4 25	11 82	20 33	4 05	11 26	0 95	6 06	0 28	0 78	0 517	−0 056	−0 156	10	36
3 08	3 26	8 89	5 06	16 50	19 58	4 57	14 90	0 90	6 38	0 50	1 63	0 538	−0 134	−0 437	13	51
4 00	3 70															
5 48	2 27	8 99	4 33	9 83	19 49	3 83	8 69	0 88	5 77	−0 10	−0 23	0 772	0 038	0 086	All	−16
6 26	3 41															
7 13	3 13															
7 43	2 74	8 99	3 78	10 36	19 34	3 47	9 51	0 92	5 80	0 12	0 33	1 048	0 215	0 589	None	36
9 00	3 55															
10 03	3 62	8 92	3 09	11 19	19 39	2 30	8 33	0 74	5 57	0 21	0 76	0 767	−0 025	−0 090	6	38

* Time is A M Symbols as in Table I Concentrations are in mM per L Q expressed as μM per min per 100 ml forearm L/G is per cent of glucose uptake accounted for by lactate production (G L)/O$_2$ is per cent of O$_2$ uptake accounted for by apparent oxidation of glucose.

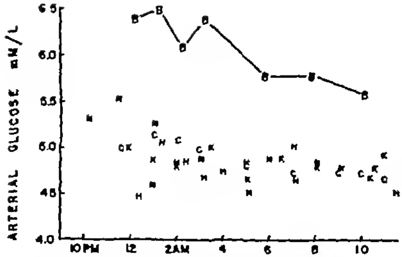

FIG. 4 ARTERIAL BLOOD GLUCOSE

B subject A. B during attack and recovery C, K and N normal subjects H, subject M. H. who failed to develop hypokalemia or clinical paralysis.

potassium movement or whether they are consequences of the disease. None of these metabolic differences appeared in subject M. H Study III, during the night in which he failed to have a spontaneous attack.

Concentration of glucose in arterial blood was definitely higher in subject A B than in normals during the night (Figure 4) and at least during the first four measurements A V glucose differences were higher than normal It has been noted previously (10) that A-V glucose differences varied directly with arterial glucose concentrations in normal subjects during the night. This relation was true also for subject A B so that the abnormally high glucose A V differences and glucose uptakes in this subject may have resulted from abnormally high delivery of glucose to muscle. Since the concentration of arterial glucose tended to decrease during the night it is not unexpected that glucose uptake tended to decrease similarly

Although interpretation of these high concentrations of glucose in arterial blood is complicated by the fact that A B had a high carbohydrate diet six and nine hours before the first blood sample was collected, even the last arterial specimen drawn 16 hours after the last meal showed higher than normal glucose concentration, 5.57 mM per L. (mean arterial glucose concentration after 16 to 18-hour fast in 24 normal subjects is 5 01 mM per L. ± 0.27 S.D)

Since uptake of glucose by skeletal muscle was at least normal the high concentration of glucose in arterial blood implies that some other organ failed to remove circulating glucose or that hepatic contribution of glucose was accelerated

Concentration of lactate in arterial blood was within normal limits during the early part of the study and rose during the later hours (Figure 5) approximately coincident with recovery from paralysis Lactate production by muscle was erratic. There is no evidence that the rise in arterial lactate concentration was owing to increased production of lactate by skeletal muscle in the forearm It is possible, however, that other muscles did contribute, since muscle power and deep tendon reflexes were examined from time to time during this period

During the night and the following morning forearm blood flow in subject A B was within the limits defined by studies of two normal subjects during the night (10) and a larger group of normal subjects during the late morning (9) Nor were there any deviations from normal in O_2 uptake in CO_2 production or in A V differences of CO_2 or O_2 content. However the arterial concentration of CO_2, initially normal fell to levels lower than those in normal subjects (mean of 24 normal subjects 21.9 mEq per L. ± 0.98 S D) (Figure 6) Although pH was not measured, this decrease in arterial CO_2 presumably represented extracellular metabolic acidosis

Mean respiratory quotients of forearm muscles in three normal subjects between the hours of 10 P M and 4 A.M were 0.75 0 84 and 0.84 In subject M H., during the same time interval

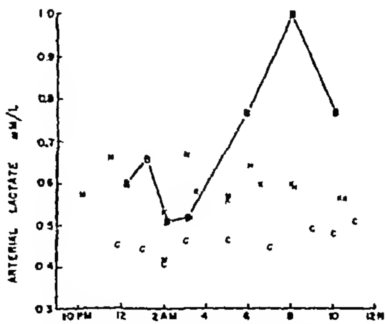

FIG. 5 ARTERIAL BLOOD LACTATE

Symbols as in Figure 4

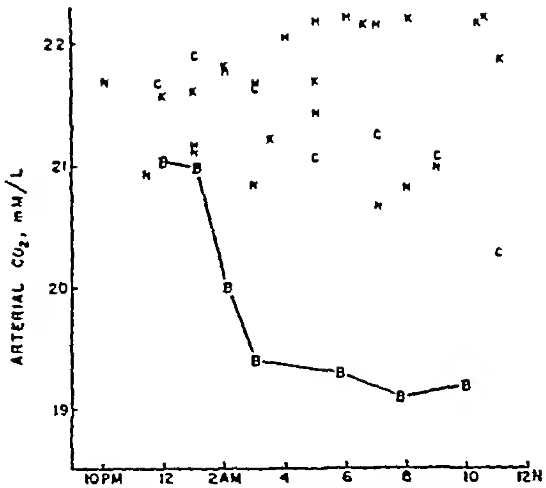

Fig 6 Arterial Blood CO_2 Content
Symbols as in Figure 4

when he failed to have an attack, the R Q was 0 82 In subject A B *during* the spontaneous attack, R Q was 1 00

The fraction of O_2 uptake by muscle which is spent in oxidation of glucose is defined by the following relation (9) 6(glucose A-V difference minus ½ lactate A-V difference)/oxygen A-V difference In contrast to data found in normal subjects during the same hours, during the early hours of the attack glucose uptake was greatly in excess of the amount needed to account for all the O_2 uptake Later, glucose uptake decreased and was sufficient to account for only a minor fraction of O_2 uptake, as was true in normals In normal subjects the fraction of O_2 uptake accounted for by glucose oxidation varied directly with arterial glucose concentration (10) This was true also in subject A B

These data do not explain the exaggerated movement of potassium between plasma and muscle seen during paralysis and recovery Although unusually rapid uptake of potassium by muscle was associated with unusually rapid glucose uptake, the direction of potassium movement reversed, and potassium moved from muscle to plasma during recovery at a time when arterial glucose concentration was still excessive and glucose uptake by muscle was still relatively large

Net efflux of potassium from cells under the influence of acidosis has been described (11) and a suggestive correlation between potassium movement from muscle and CO_2 production by muscle was found in normal subjects during the night (10) This correlation could be demonstrated for subject M H, who had no attack, but could not be demonstrated for subject A B during and following the attack

The possibility has been considered that changes in concentration of potassium in plasma may be owing to water shifts either from extravascular space or from erythrocytes into plasma To test this possibility, hematocrits were determined on paired samples of arterial and venous blood throughout the night Among seven pairs of hematocrits, mean arterial hematocrit was 44 20, range 43 1 to 45 5, mean venous hematocrit was 44 26, range 43 4 to 45 5, and mean difference between pairs of arterial and venous blood was − 0 06, range − 0 5 to + 1 0 Thus there was no evidence of net water shift into plasma

SUMMARY

1 A large net uptake of potassium from arterial plasma by skeletal muscle (the forearm) has been demonstrated a) during the development of a spontaneous nocturnal attack of periodic paralysis and b) during the development of a nocturnal attack induced by the administration of glucose and insulin

2 Movement of potassium out of skeletal muscle has been demonstrated a) during the spontaneous recovery from a nocturnal attack which developed spontaneously and b) during recovery from an attack after oral KCl administration

3 The characteristic onset of attacks during the night with spontaneous cure later in the morning seen in many of these patients may be due to exaggerations of the normal diurnal variation in net potassium movement between muscle and plasma

ACKNOWLEDGMENTS

We are indebted to the patients for their cooperation, to Dr David Grob for the opportunity of studying both patients, and to Dr Saul Farber who had previously studied patient A. B and sent him to Baltimore for electromyographic studies by Drs Grob, Ake Liljestrand and Richard J Johns Studies of A B by Drs Farber and Hugh J Carroll were reported in abstract in the J Clin. Invest., 1956, 35, 702, simultaneously with reports by Grob, Johns, and Liljestrand, J Clin Invest., 1956, 35, 708, and our own report, J Clin. Invest., 1956, 35, 747 Farber and Carroll reported that during an attack of paralysis in A. B the concentration of potassium in

plasma from antecubital venous blood was less than the concentration of potassium in arterial plasma. Their observations were made prior to our own studies of A. B and were unfortunately unknown to us at the time we performed them. We are indebted also to Miss Ellen Rogus and Mrs. Gerda von Ahlefeldt for their assistance.

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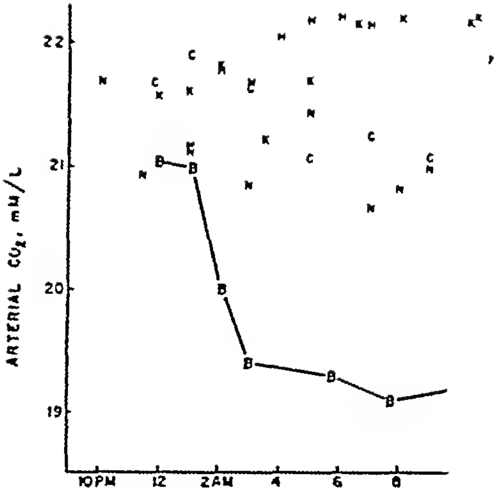

Fig. 6 Arterial Blood CO_2 Conte
Symbols as in Figure 4

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muscle seen during paralysis and recove
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muscle was associated with unusually ra
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reversed, and potassium moved from m
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glucose concentration was still excessive a
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Net efflux of potassium from cells under
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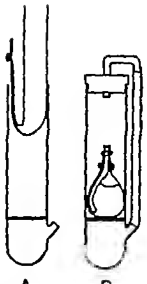

FIG. 1 THE APPARATUS USED FOR ULTRAFILTRATION OF SERUM

A The apparatus containing the knotted and inflated Visking® tubing prior to the introduction of the serum sample.

B The apparatus closed and ready for centrifugation showing the serum filled ultrafiltration sac resting on the sintered glass support.

tip of the pipet and the cellophane tubing because such contact resulted in difficulty in removing the pipet. After the serum was introduced, the cellophane bag was collapsed, and the second end was knotted at about the same height as the first end. The two knotted ends were fastened together with a rubber band, and the bag was carefully pushed down into the apparatus far enough to permit insertion of the rubber stopper To avoid tearing the bag a blunt instrument, such as a stirring rod, was used to push adherent portions of the bag away from the glass. The apparatus was then flushed for 3 minutes with a 5 per cent CO_2-95 per cent O mixture bubbled through isotonic saline and introduced through the side arm at the lower end of the apparatus. The apparatus was then closed by connecting the side arm to the glass tube in the rubber stopper at the top using Tygon® tubing thus assuring a constant atmosphere above and below the ultrafiltration sac throughout the ultrafiltration procedure (Figure 1) It was then placed in the largest cups (catalog No. 373) of an International Centrifuge, Size 2, or International Refrigerated Centrifuge, the cups having been fitted with the simple centering ring previously described (23) so that apparatus breakage was prevented while starting and stopping the centrifuge.

A temperature rise of about 8° C above the existing room temperature will occur inside the operating centrifuge in about 1 to 2 hours To keep the temperature constant during ultrafiltration in the ordinary laboratory centrifuge, the latter was fitted with a copper coil. This was connected with Tygon® tubing to the side-arm of a large glass U tube. One arm of the U tube was connected to the ordinary mixing type water spigot and the other was fitted with a thermometer Inlet water temperature was manually controlled by simply varying the amount of hot and cold water allowed to flow through the spigot into the U tube. A thermometer was fitted into the center tachometer hole of the centrifuge and the temperature inside the centrifuge was kept constant by the adjustments in the inlet water temperature.

At 23 or 37° C at least 0.5 ml. of a crystal-clear protein free (24) ultrafiltrate was collected in 3 hours when centrifuged at 2,000 rpm, while at 10 C approximately double that time was necessary If 5 ml. or more of serum were used, 1 ml of ultrafiltrate could be obtained in approximately the same times. Variations in centrifuge speed between 1,500 and 2,500 rpm do not affect the results obtained (24)

Determination of calcium. Calcium in both serum and ultrafiltrate was determined by a flame photometric technique using a Weichselbaum Varney flame photometer After protein removal (in the case of serum) the calcium was separated as the oxalate, re-dissolved, and read directly A description of the method together with a thorough study of possible variables have already been reported (25)

Determination of pH A Beckman model G pH meter was used for the pH measurements. In all instances the pH of the serum remaining in the ultrafiltration sac was measured after the ultrafiltration was carried out. In several instances, pH values of both the ultrafiltrate and the unfiltered serum residue were determined. As would be expected where the atmosphere was maintained constant over the serum and ultrafiltrate, the pH values were the same. Generally the value obtained was between 7.5 and 7.6 if the apparatus were allowed to return to room temperature (at which the equilibrium with 5 per cent CO_2-95 per cent O_2 mixture was carried out) and the pH measurement were then made.

Early in the present work it was noted that the pH tended to drift with time as the concentrated serum left in the ultrafiltration sac was being measured with a conventional asbestos fibre calomel electrode. This was virtually eliminated, however when a sleeve type calomel electrode (Beckman No. 270-71) was used.

Collection of sample Blood was collected with an ordinary syringe and a 20-gauge needle by venipuncture in the antecubital fossa. No precautions were taken to prevent loss of CO_2 (see discussion under *Effect of method of sample collection*) The ultrafiltration procedure was carried out on the same day the samples were collected.

RESULTS

In expressing ultrafiltration results some authors have used a corrected serum volume calculated from the estimated volume occupied by the proteins present. We have not used the correction but have applied the following definition to express our results

$$\% \text{ Ultrafiltrable Ca} = \frac{\text{Mg Ca/100 ml ultrafiltrate}}{\text{Mg Ca/100 ml serum}} \times 100$$

THE ULTRAFILTRABLE CALCIUM OF HUMAN SERUM
I ULTRAFILTRATION METHODS AND NORMAL VALUES [1]

By T Y TORIBARA, A. RAYMOND TEREPKA, AND PRISCILLA A. DEWEY

(From the Division of Pharmacology of the Department of Radiation Biology, and the Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, N Y)

(Submitted for publication November 26, 1956, accepted January 24, 1957)

Since the demonstration by Rona and Takahashi in 1911 (1) that a considerable portion of the calcium present in the serum is not diffusible through a semi-permeable membrane, the various calcium-fractions in the blood have been extensively studied It is now recognized that calcium exists in the serum in three distinct forms One of these, that calcium bound to proteins, comprises the non-ultrafiltrable or non-diffusible portion of the serum calcium The other two forms, ionic calcium and calcium complexed by such small anions as citrate, phosphate, and bicarbonate, are ultrafiltrable and diffusible

Ionic calcium is generally considered to be the physiologically active component of the total serum calcium (20) Recently, however, it has been suggested (21) that the "biologically active" calcium fraction of serum is different from, and probably larger than, the total ionic calcium as determined by the frog heart technique (22), approaching the value for total ultrafiltrable calcium In any event, a practical method for the routine measurement of actual ionic calcium has yet to be devised Consequently, a great deal of effort has been directed toward the development of indirect methods for its determination in man A variety of techniques has been described, but ultrafiltration methods have been most numerous because of practical and theoretical advantages over biological assays or dialysis techniques In principle, all ultrafiltration procedures utilize some type of membrane of small pore size and some means of supplying filtration pressure

The purpose of the present communication is to describe a technique for the ultrafiltration of human serum using a new, simple apparatus[2] which eliminates most of the disadvantages of previous methods Using this apparatus we have investigated the various factors which influence the ultrafiltrability of calcium in human serum and have determined the normal range in healthy human subjects A subsequent paper will describe our findings in diseased states and under experimental conditions

METHODS

Ultrafiltration apparatus and procedure The apparatus used in this work has been described very briefly in a previous publication (23) It uses seamless cellophane tubing to contain the serum with a sintered glass support for the membrane and centrifugal force to supply the filtration pressure Its principal advantages over other equipment are 1) The atmosphere and the pH can be accurately controlled within the apparatus throughout the ultrafiltration period, 2) there is no source of metallic contamination, 3) membrane breakage is virtually eliminated, 4) the apparatus is easily constructed and can be used in an ordinary laboratory centrifuge, 5) filtration pressure can be controlled by varying centrifuge speed.

The ultrafiltration procedure was carried out in the following manner

A strip of Visking Nojax Casing® (size 24/32) about 9 inches long was soaked in distilled water for 10 minutes It was wiped dry by drawing through a folded gauze sponge repeatedly until no water was visible. One end was knotted and the tubing was doubled (Figure 1) and pushed into the ultrafiltration tube with the knot up The unknotted end was opened with a sharp instrument such as a scalpel blade or small spatula and inflated by blowing into it. For the present studies, 3 ml of serum were pipetted directly into the open end of the bag Care was taken to avoid contact between the wet

[1] This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, New York.

[2] Made by sealing off the short end of a borosilicate glass straight sealing tube with coarse fritted disc (Corning No 39570, 25-mm. diameter with 20 mm disc), and adding a 6-mm glass tube at an angle near the fritted disc (Figure 1) Complete apparatus is now available from Will Corporation, Rochester, New York.

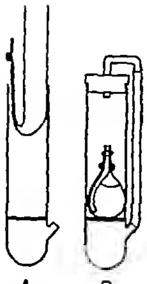

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TABLE I

Results obtained with and without precautions for loss of CO_2
Ultrafiltration temperature 23° C

Sample	Method	pH Initial	pH Final	Serum calcium Total mg%	Serum calcium Ultrafilterable mg%	Serum calcium % Ultrafilterable
R	oil	7.52	7.47	9.52	7.04	73.9
	plain	7.72	7.51	9.52	7.04	73.9
E*	oil	7.52	7.51	12.1	10.35	85.7
	plain	7.82	7.52	12.1	10.48	86.8

* Patient with carcinoma of breast and hypercalcemia.

Effect of method of sample collection

It has been reported by Hopkins, Howard, and Eisenberg (18) that when serum is equilibrated with 5 per cent CO_2–95 per cent O_2 to adjust pH before ultrafiltration, the method of collection of the blood is not particularly important Prasad and Flink (19), on the other hand, considered the method of collection and equilibration of serum highly important

Our findings are in agreement with those of Hopkins, Howard, and Eisenberg and are shown in Table I Blood allowed to clot under oil and transferred without loss of CO_2 gave results identical with those obtained when no special precautions were exercised, provided the apparatus was flushed with CO_2-O_2 mixture before the ultrafiltration procedure was carried out

Effect of duration of ultrafiltration on composition of ultrafiltrate

Since ultrafiltration is a dynamic process and concentrates the serum proteins, a criticism frequently voiced is that the calcium concentration in the ultrafiltrate may not reflect the original conditions in the whole serum This criticism may be shown to be invalid by the application of physical chemical principles to the finding of Marrack and Thacker (26) and McLean and Hastings (22) that the relationship between calcium and serum proteins may be expressed by the simple equation $Ca^{++} + Prot^{=} \rightleftharpoons CaProteinate$[3] Where a 1 1 relationship between the calcium and protein in the complex exists, these theoretical considerations show that the composition of the ultrafiltrate

[3] The thermodynamic dissociation constant of the calcium complex is

$$K = [Ca^{++}] \frac{[Prot^{=}]}{[Ca\ Proteinate]}$$

where the quantities in the brackets are activities According to this equation the ratio of bound to unbound protein is a function of the calcium ion activity in the solution. In serum of ionic strength 0 15 M, the protein concentration is about 0 001 M and is considered to contribute a negligible amount to the total ionic strength (27) Thus relatively large alterations in protein concentration must be produced before any change in ionic strength occurs Chen and Neuman (24) showed that diffusible forms of calcium (calcium chloride, calcium citrate, and calcium versenate) in 0 15 M sodium chloride move through a cellophane membrane at the same rate as water Similar behavior during ultrafiltration of serum would then cause no change in the concentration of any diffusible ions in the free water of the bulk solution, an increase in the concentration of both forms of protein, but only a negligible change in ionic strength. Since the largest factor in the determination of activity coefficients is the ionic strength, the activity coefficients of all constituents in the remaining serum would remain essentially constant during the ultrafiltration process Thus, with no change in activity coefficients, calcium ion concentration and the ratio of the two forms of protein do not change.

The above discussion would apply equally to any reaction involving a fixed number of one or more calcium ions with a single protein molecule. Thus

$$n\ Ca^{++} + Prot \rightleftharpoons Ca_n\ Prot$$

$$K = [Ca^{++}]^n \frac{[Prot]}{[Ca_n\ Prot]}$$

The ultrafiltration procedure would not change the Ca^{++} concentration and the Prot/Ca_n Prot ratio would remain constant although the concentrations of the two forms of protein change continuously

TABLE II

Successive samples during prolonged ultrafiltration. Patients with chronic renal disease and hypocalcemia

Sample	Ultrafiltration temperature	Time in hours	Serum calcium: Total mg%	Serum calcium: Ultrafilterable mg%	Serum calcium: % Ultrafilterable
K	23°	0-4	8.86	7.17	80.9
	23°	4-7	8.86	7.17	80.9
	23°	7-20	8.86	6.90	77.9
K	36°	0-3	5.58	3.81	68.3
	36°	3-7	5.58	3.81	68.3
C	23°	0-3	7.45	5.72	76.7
	23°	3-7	7.45	5.72	76.7
	36°	0-3	7.45	5.04	67.7
	36°	3-7	7.45	5.17	69.4

should be that of the non protein portion of the original serum and should not change unless the ionic strength of the solution being ultrafiltered changes significantly during the process. These considerations were confirmed by collecting successive samples of ultrafiltrate during prolonged ultrafiltration of a single 3 ml. aliquot of serum and determining the calcium content of the samples

TABLE III

Variability of ultrafilterable calcium with pH

Sample	Ultrafiltration temperature	Ultrafiltrate pH	Serum calcium: Total mg%	Serum calcium: Ultrafilterable mg%	Serum calcium: % Ultrafilterable
R	36°	7.63	9.51	6.41	67.4
	36°	8.22	9.51	5.29	55.6
B	36°	6.35	9.40	8.05	85.6
	36°	7.51	9.40	6.15	66.7
J	36°	6.35	9.27	8.60	92.8
	36°	8.18	9.27	5.31	57.3
K*	36°	7.68	11.57	8.36	72.2
	36°	8.22	11.57	6.86	59.3
Y	23°	6.52	10.13	9.26	91.4
	23°	7.63	10.13	7.76	76.4
	23°	8.40	10.13	5.82	57.3
S	23°	7.56	9.89	7.48	75.7
	23°	8.37	9.89	5.67	57.3
M*	10°	7.44	11.57	9.30	80.3
	10°	8.14	11.57	8.13	70.3

* Patient with carcinoma of the breast and hypercalcemia.

TABLE IV

Comparison of acid and carbon dioxide pH adjustment
Ultrafiltration temperature 23° C

Method of Adjustment	Adjusted pH	SERUM CALCIUM Total mg%	Ultrafilterable mg%	% Ultrafilterable
5% CO_2-95% O_2	7.55	8 14*	6 64	81.6
100% CO_2	6.48	"	7.58	93 2
Hydrochloric acid	6 58	"	7.49	92 1

* Hypocalcemia secondary to renal disease.

The ultrafiltration apparatus was re-equilibrated with the CO_2–O_2 mixture after each 0 6-ml sample was collected for analysis and the results are shown in Table II

From an original sample of 3 ml the collection of approximately 0 6 ml of ultrafiltrate for calcium analysis will result in a reduction in volume of 20 per cent in the original serum or an increase in the concentration of protein of 25 per cent In the first sample shown in the table the three successive 0 6-ml aliquots of ultrafiltrate decreased the original serum volume to less than one-half of the original volume Thus a large fraction of the serum may be ultrafiltered without an appreciable change in the composition of the ultrafiltrate, and the reduction in volume of the serum accompanying the usual ultrafiltration would not be expected to affect the results obtained

Effect of pH

The literature contains conflicting information on this subject Almost without exception, early

TABLE V

Effect of temperature on ultrafilterability of calcium

Sample	Ultrafiltration temperature	SERUM CALCIUM Total mg%	Ultrafilterable mg%	% Ultrafilterable
J	3 5°	9 36	7 96	85 1
	10	9 36	7 41	79 2
	23	9 36	6 96	74 3
	36	9 36	6 51	69 6
B	3 5°	10 27	7.46	72.6
	10	10 27	7.24	70.5
	23	10 27	6 96	67.8
	36	10 27	6 24	60.8
R	10°	9.84	7 81	79 3
	23	9 84	7 33	74 6
	36	9 84	6.44	65.5
T	10°	9.74	7.46	76.6
	23	9 74	7.26	74.5
	36	9 74	6 58	67.7

workers took no special precautions to insure constancy of pH during the process of ultrafiltration It has been reported (2, 3 10) that variations in pH during ultrafiltration have no effect on the results obtained although it was admitted that, on theoretical grounds this was unexpected (10) In any event, rigid control of pH would have been difficult since the methods most frequently utilized (Greenberg-Gunther [10] and Moritz-Updegraff [6]) had no adequate provision for maintaining a constant atmosphere to control pH throughout the entire ultrafiltration process (29) Recently, however, Hopkins Howard and Eisenberg (18) using the closed apparatus of Lavietes (28) reported a very definite effect of pH on the fraction of serum calcium appearing in the ultrafiltrate

The apparatus used in the present study also controls the atmosphere rigidly during the filtration process and we have reinvestigated the problem Our data, recorded in Table III, confirm the observation that the pH at which ultrafiltration is carried out affects significantly the fraction of calcium that is ultrafiltrable. Analysis of these data shows that the per cent of ultrafiltrable calcium changed from 1 5 to 2 5 per cent per 0 1 pH unit in good agreement with values calculated from data previously reported (26 18 30)

The lowering of pH was accomplished by equilibrating the serum in the ultrafiltration apparatus with 100 per cent CO_2 The high pH's were obtained by ultrafiltering serum with no carbon dioxide equilibration. Hopkins Howard and Eisen

TABLE VI

Ultrafilterable calcium values for normal human serum at various temperatures (All samples equilibrated with 5 per cent CO_2—95 per cent before ultrafiltration)

Sample	Sex	Ultrafiltration temperature	Total Calcium*	Ultrafilterable Calcium*	% Ultrafilterable
W B	M	36°	9.86	6.54	66.2
J V	M		9.73	6.38	65.6
B.J	M		10.30	6.27	60.9
R.T	M		9.60	6.20	64.6
T T	M		9.26	6.38	68.9
B B	M		10.22	7.16	70.0
E L.	M		9.53	6.80	71.4
H O	M		10.12	6.17	60.9
K.F	F		9.54	6.20	64.9
B.J	F		9.36	6.51	69.6
R B	F		10.27	6.24	60.7
J B	F		9.27	6.18	65.8
D T	F		9.45	6.42	67.9
					60.7 – 71.4**
B.S	M	23°	9.89	7.48	75.6
I.F	M		10.15	7.76	76.4
T T	M		9.74	7.26	74.6
B T	M		9.84	7.33	74.6
R B	F		10.27	6.96	67.8
B.J	F		9.36	6.96	74.4
K.F	F		9.83	7.26	73.8
					67.8 – 76.4**
W S.	M	10°	10.18	6.98	68.7
P C	M		9.94	7.44	74.8
J.C	M		10.00	7.54	75.4
P S	M		9.70	6.92	71.3
E.F	M		10.30	7.92	76.8
O K	M		10.22	7.60	74.4
A C	M		9.64	7.63	79.1
E.L.	M		9.53	7.57	79.4
P D	F		10.10	7.51	74.4
J C.	F		10.06	7.52	74.6
B.D	F		10.30	7.78	75.5
J.S	F		9.96	7.45	74.8
D B	F		10.00	7.58	75.8
M.R	F		9.40	7.22	75.8
B L.	F		9.84	8.00	81.4
R.B	F		10.27	7.24	70.4
					68.7 – 81.4**

* Values in mg%
** Range for group.

of ultrafiltrable calcium in human subjects during a single day, however, are remarkably constant (32).

In Table VIII is recorded the per cent ultrafiltrability or diffusibility of the calcium of normal human serum calculated from the data of previous authors. It is immediately apparent that with the exception of von Meysenbug, Pappenheimer, Zuker, and Murray (2), Rona and Melli (4), Nicholas (13) and Kirk and King (5), earlier workers obtained results significantly lower than our values and those of the two most recent authors (18, 19).

From the previous discussion on the effect of pH on the quantity of ultrafiltrable calcium it seems most probable that failure to control the pH during ultrafiltration was predominantly responsible for the consistently lower values obtained by these early workers. In this connection, it is of interest that the results of von Meysenbug, Pappenheimer, Zuker, and Murray (2) and of Rona and Melli (4) were obtained by compensation dialysis in a closed system where the pH was probably maintained constant throughout the procedure. The original Rona and Takahashi investigations of 1911 (1) were done by essentially the same method. Their value for the diffusible serum calcium of various domestic animals was between 65 per cent and 75 per cent! Nicholas (13) and Kirk and King (5) took no special precautions regarding pH control but the apparatus they used was closed during the entire filtration and positive pressure was maintained over the serum with nitrogen or air.

DISCUSSION

Our data indicate that, with a normal total serum calcium concentration of about 10 mg. per cent, 6 to 7 mg. per cent of this calcium is ultrafiltrable. McLean and Hastings (22), by their frog heart technique, concluded that about 1.3 mM per L. (5.2 mg. per cent) of "ionic" calcium exists in normal serum. Calculations based on the actual normal serum concentrations of citrate, phosphate, and bicarbonate, activity coefficients, and the reported pK's of the calcium complexes of these anions reveal that about 0.3 mM per L. (1.2 mg. per cent) of calcium exists in normal serum in the form of these known complexes (33). The sum of these two values, 6.4 mg. per cent, is remarkably similar to the mean value of our experimentally determined range for normal total ultrafiltrable calcium and closely approaches the value for "biologically active" calcium suggested by the data of Yendt, Connor, and Howard (21).

Variations in the pH of the blood in human beings *in vivo* seldom extend more than 0.2 pH units above or below the 7.35 to 7.40 range even in pathological states involving alterations in acid-base balance. From our data this would mean only a minor alteration in the actual quantity of ultrafiltrable calcium, well within the 10 per cent range found in normal human subjects. In acute conditions, however, such as hyperventilation tetany, a sudden minor shift in the quantity of available calcium may well be highly important. It has been reported that in hyperventilation tetany, total serum calcium and total ultrafiltrable calcium do not change significantly (34, 35). However, the actual alterations in the quantity of calcium involved due to pH changes in the physiological range are small enough so as to lie near or within the limits of accuracy of all the experimental methods for estimating total and ultrafiltrable calcium.

The observed effect of temperature on the quantity of ultrafiltrable calcium in human serum was somewhat surprising. Most complexes become more stable at lower temperature, but we found that more calcium was ultrafiltrable as the temperature at which the procedure was carried out was decreased.

The only bits of corroborative evidence for this temperature effect we were able to find were in the work of McLean and Hastings (22), Lavietes (28), and Marrack and Thacker (26). In 1926, the latter authors, by dialysis of protein solutions at room temperature and at 37° C, concluded that values for non-diffusible calcium of serum would be lower if the determinations were made at room temperature. Lavietes, in 1937, described his anaerobic ultrafiltration apparatus and referred to the fact that the quantity of calcium filtered varied with temperature but no data were given. McLean and Hastings noted that the amount of "ionic calcium" was about 0.15 mM per L. (0.6 mg. per cent) higher at 15° C than at 25° C. Although questioning the significance of this observation, they did calculate by extrapolation that calcium ion concentrations would be 0.8 mg. per cent lower

at 38° C than those observed at room temperature if this temperature effect were real. That is with an average normal serum calcium of 10 mg per cent the concentration would be about 8 per cent lower This value agrees very well with our data on normal values at different temperatures recorded in Table V

The exact mechanism responsible for this temperature effect is not clear It may be that in the binding of calcium to protein a weaker bond is obtained at lower temperatures but Katz and Klotz (36) claim that the binding of calcium to bovine serum albumin is independent of temperature from 0° C to 25° C A change in pH is apparently not involved. We have found that the pH in an aliquot of serum measured at room temperature, after 5 per cent CO_2–95 per cent O_2 equilibration at that temperature, did not change significantly when the pH was remeasured after heating to 36° C.

One other possible explanation for the phenomenon of decreased protein bound calcium at the lower temperatures is suggested when one considers the binding of calcium by other anions in serum such as citrate, phosphate, and bicarbonate. The relationship between the latter and ionic calcium and calcium bound to protein can be expressed by the equation $CaX \rightleftharpoons Ca^{++} \rightleftharpoons CaProt$ where X refers to these ultrafiltrable anionic complexers of calcium If the temperature coefficients of the filtrable complexes of calcium were greater than those of the protein complexes the net effect of a lowering of temperature would be to have more of the calcium in the form of these ionic complexes and a resultant increase in ultrafiltrable calcium as found The data of Katz and Klotz (36) who found that the binding of calcium ions by bovine serum albumin was independent of temperature lend support to this explanation. If this explanation is correct a net *decrease* in the actual ionic form of calcium would occur as the ultrafiltration temperature is lowered although total ultrafiltrable calcium increases That is more of the calcium ultrafiltered would be in an associated form Thus it would appear that the McLean and Hastings frog heart method which also responds to a lowering of temperature with an apparent increase in ionic calcium measures more than just the ionic calcium in the test solution It is clear that further work with pure proteins is necessary to elucidate the exact the temperature effect observed.

SUMMARY

1 A new simple ultrafiltration appara scribed and the procedure for the ultr of human serum is outlined.

2 The importance of the method of se lection, duration of filtration pH and te was investigated The ultrafiltration re affected considerably by pH and tempe

3 For healthy human subjects the ultrafiltrable calcium at physiological temperature was found to be 60 to 70 p the total serum calcium The results o authors are reviewed and compared with ent findings

4 The quantitative distribution of t calcium fractions in human serum and t cance of the observed effect of pH and ture on the ultrafiltrability of calcium are

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DISCUSSION

All the results reported here fit the assumption that factors for d (diffuse) and s (single) hemoglobin patterns segregate as simple Mendelian autosomal alleles. The results of crosses of d × s (Table I) in the first hybrid (F_1) in the second hybrid generation (F_2) (Table II) as well as in the backcross generations (Tables III and IV) might be interpreted as indicating that d is dominant over s. Although the method of paper electrophoresis employed did not permit distinction of hemoglobin patterns of animals carrying the factors for both the d and the s type from the hemoglobin pattern of animals carrying the factor for d only, it is conceivable that the hemoglobin composition of d/s animals might be distinguished from that of animals homozygous for d by other methods. Further experiments are planned to elucidate this point. The decision between dominance and co-dominance of d and s can therefore not be made at present.

The distribution of hemoglobin patterns of F_2 animals and of backcrosses of both F_1 and F_2 animals to the parent strain with the single type hemoglobin pattern is entirely consistent with the assumption that d and s behave as alleles segregating in the expected ratios.

Comprehensive data on the genetic aspects of human hemoglobins have been summarized by Neel (4); the genetic aspects of hemoglobins in man are somewhat different from those found in mice. Human hemoglobins are controlled genetically by a group of factors some of which behave as alleles; it seems that each allele is responsible for the presence of one particular hemoglobin. With respect to human hemoglobins, then, an individual homozygous for any one of these genetic factors shows the presence of pre-

TABLE III

Results of backcrosses of F_1 generation from d × s to s strains

Exp	Origin of F_1 parent	BC parent	Offspring d	Offspring s
1	from BALB (d) × Spl (s)	Spl (s)	25	21
3	from Fu (d) × ur (s)	ur (s)	16	23
5	from C_3H (d) × Ki (s)	Ki (s)	8	[illegible]
	All F_1 from d × s backcrossed to s		49	53

Numbers of experiments correspond to original F_1 crosses as listed in Table I

TABLE IV

Results of backcrosses of F_2 generation to s strains

Exp.	Origin of F_2 parent	Hemoglobin pattern of parent	Number of animals tested	BC parent	Offspring d	Offspring s
A						
1	F_2 from DBA (d) × Ki (s)	d	2	Ki (s)	13	0
2	F_2 from BALB (d) × Spl (s)	d	1	Spl (s)	3	0
3	F_2 from Fu (d) × ur (s)	d	3	ur (s)	17	0
5	F_2 from C_3H (d) × Ki (s)	d	1	Ki (s)	4	0
	A *all non segregating F_2 d*		7 = d/d	s	37	0
B						
1	F_2 from DBA (d) × Ki (s)	d	4	Ki (s)	16	13
2	F_2 from BALB (d) × Spl (s)	d	3	Spl (s)	11	9
3	F_2 from Fu (d) × ur (s)	d	1	ur (s)	11	1
4	F_2 from C_3H (d) × Spl (s)	d	1	Spl (s)	1	2
5	F_2 from C_3H (d) × Ki (s)	d	3	Ki (s)	6	8
	B *all segregating F_2 d*		12 = d/s	s	45	33
C						
1	F_2 from DBA (d) × Ki (s)	s	6	Ki (s)	0	31
2	F_2 from BALB (d) × Spl (s)	s	2	Spl (s)	0	13
3	F_2 from Fu (d) × ur (s)	s	1	ur (s)	0	2
4	F_2 from C_3H (d) × Spl (s)	s	1	Spl (s)	0	4
5	F_2 from C_3H (d) × Ki (s)		5	Ki (s)	0	18
	C *all F_2 s*		15 = s/s	s	0	68

Numbers of experiments correspond to original F_1 crosses as listed in Table I

Effect of patient's plasma and serum on clotting defect of a case of congenital deficiency of SPCA

Lyophilized samples of the patient's plasma and serum were sent to Dr Benjamin Alexander who found that the plasma of his patient (1) (congenital deficiency of SPCA by prior definition) and the plasma of our patient were mutually corrective in the prothrombin time test. Dr Alexander has informed us that the thromboplastin generation test is normal in his patient (43) He has also found that both patients appear to lack "proconvertin" as measured by Owren's technique (6), and that a mixture of equal parts of the plasmas of the two patients appears to have 50 per cent "proconvertin" activity in the Owren assay These data suggest strongly that the defects in the two patients should be considered distinct

Effect of patient's plasma and serum on clotting defect of case of Crockett and associates

Crockett and associates (25) studied a patient (H H) with a congenital clotting defect This patient had a prolonged prothrombin time which was corrected by normal serum It can be seen (Table VII) that a normal prothrombin time was not obtained when equal parts of the plasma of the above patient and the plasma of R S were mixed, although mixture with normal plasma was successful with both patients It was shown also that the two sera were not mutually corrective in the thromboplastin generation test, an equal mixture of each being inactive. The defects in the two cases therefore can be considered the same

TABLE VII

The effect of patient's plasma of normal plasma and the plasma of a previously described patient with a clotting defect

Plasma			Prothrombin time (*sec.*)
R. S.	H. H.	Normal	
		0.1	12.2
	0.1		38
0.1			60
0.05	0.05		44
	0.05	0.05	13.4
0.05		0.05	13

DISCUSSION

The direct mixing of the plasmas of our patient and Alexander's with mutual correction of the prolonged prothrombin times, plus the completely different action of the two in the thromboplastin generation test indicate that the two defects are not identical Thus, a patient diagnosed as having SPCA deficiency and one diagnosed as having proconvertin deficiency have different defects This throws open the whole question of the action of these factors and requires that the literature be re-evaluated in the light of this finding The puzzling inconsistencies in Table I with regard to thromboplastin generation and "Stypven" action are probably explained by heterogeneity of the cases

It would be interesting to know the effect of "Stypven" on the plasma of Alexander's patient The fact that "Stypven" failed to correct the defect of both our patient and Crockett's, yet corrected the defects of Hicks' (16) and Jenkins' (38) patients with Factor VII deficiency, Hjort, Rapaport, and Owren's with hypoproconvertinemia (39) and Telfer, Denson, and Wright's with "Prower factor" deficiency (23) suggests that "Stypven" might prove useful in categorizing bleeders with a prolonged prothrombin time due to absence of one of the "stable" factors

It is of great interest that the Stuart factor is required for "thromboplastin" formation in the thromboplastin generation test while SPCA is not (43) This parallels the finding of delayed prothrombin utilization in our patient (9, present paper) and normal prothrombin utilization in Alexander's (1) The abnormal prothrombin utilization in our patient is confirmed and explained by our *in vitro* experiments In the experiment shown in Figure 1, thrombin evolution from prothrombin of the patient's plasma in a 2-stage prothrombin assay was markedly abnormal unless a serum factor was added We have found also that the yield of thrombin from partially purified human prothrombin (29) is proportional to the concentration of "Product II," in additional experiments not included in the present communication. Thus there appears to be a direct relationship between Stuart factor concentration and activity of Product II on the one hand, and concentration of Product II and the yield of

thrombin from prothrombin on the other The prothrombin utilization defect in our patient appears secondary to defective formation of blood thromboplastic activity

The experiments with the washed sediments (Table V) show clearly that the Stuart factor is as necessary as AHF and PTC for the formation of the early intermediate, Product I, which appears to unite in some manner with platelets to form a sedimentable thromboplastin Product II The sedimentable Product II, after a wash in saline, gives the same normal "prothrombin time" with the patient's plasma as with normal plasma suggesting from another direction that the patient's defect in conversion of prothrombin to thrombin is the reflection of a defect in the formation of a "complete" blood thromboplastin. The facts that washed normal platelets alone do not correct the patient's defect while the washed Product II prepared with normal serum does correct it, seem to imply that the Stuart factor in the Product II sediments is more closely bound to platelets than mere occluded plasma.

Retrospectively it would appear that the chief reason for the assumed identity of Factor VII SPCA and proconvertin has been the wide use of assays of the Koller (3) and Owren (4) types The substrate for both methods consists of plasma filtered through asbestos This substrate is known to contain most of its original prothrombin but has been thought to be deficient in only a single accessory factor Alexander (43) has found that the plasmas of both his patient and ours appear to lack proconvertin by the Owren method (4) while an equal mixture of the two has approximately half the activity of normal plasma. This suggests that the asbestos filtration step has removed both SPCA and Stuart factor

It is interesting that a "brain extract co-factor" which is not the Stuart factor is depressed early in dicoumarol therapy There is a short period in other words early during dicoumarolization when the prothrombin time is lengthened yet Factor V and prothrombin concentration are high, Stuart factor and PTC levels are not significantly reduced and the thromboplastin generation test is normal Later during therapy the Stuart factor becomes reduced along with PTC (44–46) and prothrombin (47)

The dicoumarol experiments raise the question whether the Stuart factor is identical with the postulated new factor, Factor X (48) since the Factor X effect was first noted in dicoumarol plasma Factor X appears to be less stable than the Stuart factor since Factor X is said to disappear in a few hours at room temperature (48) and the Stuart factor is stable much longer under similar conditions Moreover Factor X in concentrations varying between 1 per cent and 100 per cent is thought to affect the velocity of blood thromboplastin formulation but not the final yield (48) although in concentrations lower than 1 per cent, thromboplastin generation may be almost impossible (48) Our experiments show that the amount of coagulant activity produced in the thromboplastin generation test is related directly to Stuart factor concentration It would appear that the Stuart factor is not the same as Factor X.

The pH and storage stabilities of Stuart factor are pronounced for a clotting factor and almost identical with those shown for SPCA by de Vries Alexander and Goldstein (2) Since it has been demonstrated that Alexander's patient and ours have different defects this poses a serious problem in interpretation. There are at least two possible explanations for the similarity of the properties of SPCA and Stuart factor Either the two factors have very similar physical and chemical though different physiological properties, or de Vries and associates (2) were measuring Stuart factor in their SPCA assay rather than the factor which their patient (later described) was found to lack It would be very interesting to compare the results obtained if their experiments were repeated, using the genetically deficient SPCA plasma as the test substrate alongside the original assay

Heretofore, it has been agreed that the factors which are clearly essential for a normal thromboplastin generation test (AHF and PTC) have no effect on prothrombin time Also the stable factor essential for a normal prothrombin time (SPCA Factor VII) has been recognized as having an equivocal relation to thromboplastin generation The Stuart factor appears to be necessary for both a normal prothrombin time and for a normal thromboplastin generation test This is disturbing because the characteristics of Stuart factor cut across our usual thought categories It

raises the question whether the Stuart defect is not, in reality, a double deficiency? This question cannot be answered categorically at present, because of the omnipresent possibility of undescribed factors However, it seems unlikely for two reasons It has been shown previously that Stuart's plasma (then thought to be SPCA-deficient) corrects the prolonged partial thromboplastin time of plasmas from classic hemophilia, PTC-deficiency, Ac-globulin deficiency, and PTA deficiency (49) Alexander has shown that it also corrects SPCA deficient plasma (43), and it does not have the characteristics of Hageman factor (50) or Factor X (48) The hypothesis of double deficiency would imply under these circumstances that the plasma is deficient in *two new* factors The principle of economy of hypotheses suggests that it might be wise to invoke only one new factor at this time Secondly, if the conventional genetic assumption that each of these deficiencies results from a mutant gene at a specific and unique locus is made, the probability of a double deficiency can be shown to be exceedingly small

It is not possible at present to decide with certainty which of the reported cases of SPCA, Factor VII and proconvertin deficiency probably match Alexander's patient and which ours Retesting all of the patients with both the thromboplastin generation test and "Stypven" would be helpful However, mutual exchange and cross-matching in several clotting systems appears to be the ultimate test Of the patients in the literature (other than Crockett's), our patient's defect more nearly resembles that of de Vries' patients (15), Stefanovic's (20), Newcomb's (22) and Telfer's (23), the ones having abnormal thromboplastin generation tests However, and this may be crucial, the Prower defect of Telfer's patient is corrected by "Stypven" in marked contrast to both our patient and Crockett's This may well mean that the Stuart and Prower defects are different The absence of tests with "Stypven" does not allow one to speculate further about the others It is possible also that Quick, Pisciotta, and Hussey (13) have cases of both Stuart factor and SPCA deficiency amongst their patients with prolonged prothrombin times but showing mutual correction At the moment, this possibility is obscured by the lack of two-stage prothrombin data as well as thromboplastin generation and "Stypven" tests We would like to suggest, and are prepared to cooperate ourselves, that the workers who have reported cases of "stable factor" deficiency exchange lyophilized samples of plasma and serum in an attempt to categorize these patients exactly

CONCLUSIONS

1 A patient was re-studied who had been diagnosed previously as hypoproconvertinemia He had an abnormal thromboplastin generation test, and his defect was not corrected by "Stypven"

2 The deficient factor was shown not to be SPCA by cross-matching and is being called the *Stuart factor* after the patient's surname

3 Stuart factor has been found to be essential for the formation of "blood thromboplastin"

4 Stuart factor has unusual actions, being necessary early in "blood thromboplastin" formation and required for optimal activity of brain, lung, and platelet thromboplastins, cephalin and "Stypven"

5 The concentration of Stuart factor has been found to be high early in dicoumarol therapy, despite a prolonged prothrombin time, but to be diminished later

6 Stuart factor is relatively heat and pH stable

7 Stuart factor can be separated from platelets by a single saline wash, but is not removed from the sedimentable coagulant, "Product II," by a similar procedure

8 Assay procedures for "proconvertin" and "Factor VII" using asbestos-adsorbed plasma as substrate are probably sensitive to changes in the levels of both SPCA and Stuart factor

9 The hemorrhagic state(s) previously classified as congenital "hypoproconvertinemia," or "SPCA deficiency" or "Factor VII deficiency" are probably not identical diseases There are at least two separable conditions included in this group

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STUART CLOTTING DEFECT II GENETIC ASPECTS OF A NEW HEMORRHAGIC STATE

By JOHN B GRAHAM, EMILY M. BARROW AND CECIL HOUGIE [1]

(*From the Department of Pathology University of North Carolina Chapel Hill N C*)

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The literature is ambiguous regarding patients deficient in the clotting factor variously known as 'SPCA' "proconvertin, and 'Factor VII" Sera from some patients are normal in the thromboplastin generation test those from others are abnormal Viper venom corrects the clotting defect in some cases but fails to do so in others Despite these contradictions, it has been generally assumed that the terms are synonyms for a single clotting factor with unique characteristics

In our preceding communication on the patient R S (1) previously reported by others as hypoproconvertinemia (2), we pointed out that the assumption of identity must be incorrect since the plasmas of our patient and the SPCA deficient patient of Alexander, Goldstein Landwehr, and Cook (3) were mutually corrective while our patient's plasma failed to correct that of the patient of Crockett, Shotton Craddock and Leavell (4) This finding implied the existence of at least two $BaSO_4$ adsorbable clotting factors whose lack prolongs the prothrombin time. We showed that, in contrast to certain other cases in the literature (5–8), the defect in our patient's plasma could not be corrected by Russell viper venom and that his serum was inactive in the thromboplastin generation test In this last respect our patient's defect resembled that of some of the previously reported cases (8–11) but differed from certain others (5 6 12–14)

The factor deficient in our patient is being referred to as the Stuart factor after the patient's surname We wish to emphasize by this nomenclature that only by cross matching his plasma with that from other similar patients can an identity be definitely established The properties of Stuart factor and its role in blood coagulation were the subject of our earlier communication (1) In this paper the studies on the relatives of our patient will be reported Our genetic studies indicate that the Stuart defect is inherited as a highly penetrant, incompletely recessive autosomal characteristic.

MATERIALS AND METHODS

Blood for study was obtained from certain members of the family on three separate occasions. On the first field trip blood was obtained from the proband, his wife, and two of his sons On the second field trip blood was obtained during the course of a single day from all the persons shown in Table I On the third trip blood was obtained from the proband, his wife, and his daughter It was established on the first trip that the plasma and serum of the proband's wife did not differ significantly in concentration of Stuart factor from several, presumably normal laboratory workers. Henceforth, the wife was used as the control subject, in the belief that a field control was required to cover the manipulations of venipuncture, transportation and storage. On all occasions blood was obtained by the two syringe, silicone technique and was centrifuged in a portable Servall centrifuge in siliconed centrifuge tubes.

Plasma was pipetted immediately from the centrifuge tubes into storage tubes prothrombin time was determined at once on a sample and the remainder was quickly frozen and stored at – 70° C. The following day prothrombin times and Stuart factor assays were performed on all samples of plasma in our own laboratory

Serum was obtained from the members of the family at the same venipuncture as plasma. Whole blood was placed in clean, non-siliconed centrifuge tubes. Tubes were stoppered and the blood allowed to clot, precautions being taken to prevent hemolysis. The clotted blood remained at automobile and room temperature for 18 to 21 hours before centrifugation. The serum was expressed by centrifugation in our own laboratory the next day and tested for residual prothrombin and thrombin. When a serum sample contained detectable prothrombin or thrombin it was placed in a 28° C waterbath until the clotting time with thromboplastin plus fibrinogen was greater than 300 seconds and it did not clot fibrinogen in 10 minutes. This additional incubation was required in only a few cases notably the proband's. All samples of serum were frozen and stored at – 20 C, including the control serum from the proband's wife. All thromboplastin generation tests were performed on the second and third day after obtaining the serum. The same Al(OH) adsorbed normal plasma and 0.03 per cent

[1] Present address Department of Clinical Pathology University of Virginia, Charlottesville, Virginia.

TABLE I

The plasma and serum Stuart factor levels of fourteen members of the Stuart kindred

Number	Relation to proband	Per cent of Stuart factor: Prothrombin time method	Thpln generation method	Average	Probable genotype
IV-28	Proband	0*	0*	0	Homozygous abnormal
III-11	Mother	47	40	44	Heterozygous
III-23	Paternal uncle	44	45	44	Heterozygous
III-25	Paternal aunt	52	37	44	Heterozygous
V-37	Son	32	20	26	Heterozygous
V-38	Son	32	22	27	Heterozygous
V-39	Son	11	31	21	Heterozygous
V-40	Son	32	26	29	Heterozygous
V-41	Daughter	44	60	52	Heterozygous
III-3	Maternal aunt	100	100	100	Homozygous normal
III-5	Maternal uncle	93	80	86	Homozygous normal
III-21	Paternal uncle	100	100	100	Homozygous normal
IV-7	Aunt by marriage	100	100	100	Homozygous normal
IV-26	Wife	100	100	100	Homozygous normal

* One to three per cent according to Lewis, Fresh, and Ferguson (2)

cephalin suspension (in lieu of platelets) were used in all generation tests

Stuart factor assays

Plasma method The assay procedure was based on a comparison of the correction by control and test plasma of the proband's prolonged prothrombin time. The plasma of the proband (prothrombin time 60 to 85 seconds) was the substrate for the test. Normal plasma (prothrombin time 12 to 14 seconds) was diluted with the atient's plasma 1:2 through 1:16 Prothrombin time 15) was determined on the various mixtures in tripliate and the values at each dilution were averaged. A alibration curve was constructed by plotting prothrombin ime against concentration of the Stuart factor, i e, the ercentage of normal plasma in each mixture, the 1:2 ilution representing 100 per cent.

An unknown plasma was assayed after being diluted 2, 1:4, and 1:8 with patient's plasma. Prothrombin imes were performed on each dilution in triplicate and veraged. Per cent Stuart factor was interpolated from he calibration curve for each dilution of the unknown lasma The percentage value from the 1:4 dilution was oubled, the value from the 1:8 dilution quadrupled, and oth were averaged with the value from the 1:2 dilution. The final average value for each plasma is shown n Table I

The arbitrary 100 per cent value in this test is actually quivalent to 50 per cent Stuart factor An unknown lasma with 50 per cent Stuart factor is, therefore, one hich diluted 1:2 with Stuart's plasma, has a clotting ime equal to that of the normal control diluted 1:4 A typical dilution curve on normal plasma, November 7, 1955 was as follows 1:2(100%)–14.4 secs, 1:4 50%)–16.5 secs, 1:8(25%)–19.6 secs, 1:16(12.5%)–4 secs)

Serum method The Stuart factor content of an unknown serum was determined in the thromboplastin generation test (16) after it had been established that the proband's serum was only about as effective as saline in this test (1) Normal serum was diluted in a serial two-fold fashion 1:2 through 1:8 with proband's serum prior to diluting 1:10 with normal saline for use as the serum source in the generation test. The calibration curve was constructed by plotting the minimum clotting time at each dilution (at whatever incubation time) against Stuart factor concentration, i e, the per cent normal serum in the mixture.

Unknown sera were assayed by diluting 1:2, 1:4, and 1:8 with patient's serum then 1:10 with saline and determining the minimum clotting time in the thromboplastin generation test (at whatever incubation time) Per cent Stuart factor in each dilution of each unknown was determined by interpolation from the calibration curve. The percentage Stuart factor obtained at the 1:4 dilution was multiplied by 2 and that at 1:8 by 4 All three percentage values were averaged and are the averages shown in Table I

In this assay the 1:2 dilution of normal serum with patient's serum was arbitrarily designated 100 per cent on the calibration curve. A value of 50 per cent or less for an unknown serum implies that it had the same or less activity than normal plasma carried one dilution step further (A typical dilution curve of control serum with patient's serum on November 29, 1955, showed the following minimum clotting times 1:2(100%)–14.5 secs, 1:4 (50%)–17 secs, 1:8(25%)–22 secs)

PEDIGREE

The proband belongs to a large kindred living in the Blue Ridge mountains of the northwestern corner of North Carolina and nearby southwestern Virginia Information was obtained on 164 members of the family, approximately 100 still reside

within a 30-mile radius of West Jefferson North Carolina.

The pedigree is shown in Figure 1 The proband (IV-28) a moderately severe bleeder and symptomatic since birth, had not required transfusions until December, 1955, at age 36 His relatives were quite dogmatic that only he should be considered a bleeder Closer questioning of his wife however disclosed that one of his sons (V 39) seemed to have unusually frequent nosebleeds, and that the buttocks of his daughter (V-41) often showed persistent bruises after disciplinary spanking No history of even a mild bleeding tendency could be elicited from other members of the kindred

The proband works as a tenant farmer, farm laborer and lay preacher in the periods between hemorrhagic crises, never dangerous until the latest one. Exacerbations have been of such frequency and irregularity that he has been unable to retain regular employment and has found it difficult to support his family His chief symptoms have been excessive bleeding from small cuts occasional hemarthoses and persistent anemia.

It can be seen in Figure 1 that our proband was born of a consanguineous union His mother (III 11) and father (III 12) were related to each other as aunt and nephew a not unknown mating type 40 years ago in this isolated mountain area. It should be noted also that there is an other but less close consanguineous mating in the pedigree (III 5 to IV-7) in which the abnormal genes were probably absent as judged by assay procedures and 13 apparently normal children.

We assayed for the Stuart factor both plasma and serum from the 14 persons needed to establish a genetic hypothesis Those available included the proband s mother children and wife, two of the mother s siblings and three siblings of his deceased father The results are shown in Table I It can be seen that the levels of Stuart factor found with the two procedures agree fairly well If the values from the two procedures are averaged they fall clearly into three distinct groups There is an intermediate class between the very low level of the proband (1 to 3 per cent according to Lewis and associates [2]) and the normal persons, such as his wife The mean value for Stuart factor in this intermediate group is 36 per cent with a standard deviation of 9 6 per cent. It is logical therefore, to hypothesize that the proband is homozygous for an abnormal autosomal gene, that the intermediate class is heterozygous and that the persons with normal levels are homozygous normal

This hypothesis can be tested for internal consistency by analyzing the distribution of the heterozygous and normal individuals

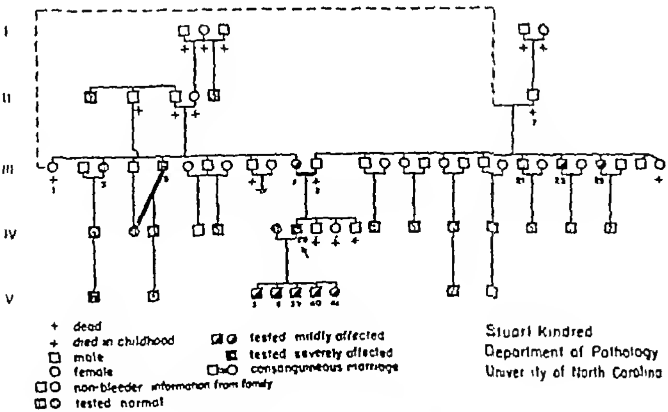

FIG. 1 THE STUART KINDRED

1 The mother of the proband, all his children and two siblings of his (deceased) father are heterozygous as classified by our tests
2 Both father and mother of the proband have normal siblings
3 The proband's wife is normal, and there are no homozygous children
4 The heterozygous siblings of the proband's father, who did not mate consanguineously, do not have clinically affected children

All these facts are consistent with the hypothesis that the proband is homozygous for an incompletely recessive autosomal gene (*st/st*), his mother, two of his father's siblings and all his children are heterozygous (*St/st*), and his maternal aunt, maternal uncle, paternal uncle, aunt by marriage and wife are homozygous normal (*St/St*)

Another hypothesis which might fit these data is that an autosomal gene for the Stuart defect is completely expressed in some individuals in the heterozygous condition (the proband) but very slightly expressed in others (his mother and children) This can be rejected by observing that all five of the proband's children would be required to have received the abnormal gene from their father If his wife, genetically unrelated and normal by test, were in fact normal (a reasonable assumption), the probability that every child of a heterozygote also would be heterozygous is 1 in 32 or between 3 per cent and 4 per cent, a sufficiently low probability for rejection

A sex linkage hypothesis can be excluded by noting that the mother and all the children of the proband, four of whom are males, have a mild form of the condition which is severe in him, and that his wife is normal It is interesting in this connection to note the family history of another case of Stuart factor deficiency, H H, previously reported as a case of congenital hypoprothrombinemia by Crockett and associates (4) and shown to have Stuart factor deficiency in our earlier publication (1) This second patient is female and her father, mother, and sister appeared normal clinically

Our hypothesis implies that in the Stuart kindred the sisters, III-1 and III-11, were probably heterozygous at an autosomal locus and that the man, now deceased, who was III-1's son and III-11's husband was also heterozygous The consanguineous mating of III-11 and III-12 produced at least one homozygote, the proband (IV-28) No conclusions can be drawn about the proband's siblings since two died in infancy and the third died of a cerebral vascular accident which might have resulted either from hemorrhage or thrombosis

Under our hypothesis, one-half of the members of generation III and all of the proband's children should be heterozygous Reference to Figure 1 will show that this prediction is borne out precisely Six members of the third generation and all the children were tested Three of the six adults were clearly heterozygous as were all the children These facts mean that, assuming our hypothesis to be correct, the abnormal gene is not only incompletely recessive but is also highly penetrant

Finally, it should be noted that the persons with intermediate levels of the Stuart factor and scored as heterozygous, had 1½ to 3-second prolongations in the classical prothrombin time test and minimum clotting times in the routine thromboplastin generation test (serum phase) 2 to 3 seconds longer than control sera

DISCUSSION

The data outlined above establish beyond reasonable doubt that Stuart factor deficiency is inherited in this pedigree in an incompletely recessive autosomal fashion, expressed in its most severe form in the homozygote The homozygote has only a moderately severe hemorrhagic diathesis as was pointed out earlier by Lewis, Fresh, and Ferguson (2) The heterozygotes consider themselves normal, but close questioning discloses that some have a mild tendency to bleed excessively It is of interest that heterozygosity for this mutation can be detected by appropriate tests From the theoretical standpoint this finding illustrates again the axiom that every gene has an effect and implies that gene frequency studies based on heterozygote counts are possible The data of Brink and Kingsley (17, 18) and Lewis and Ferguson (19) showing similar effects in

persons heterozygous for Factor V deficiency have, perhaps similar implications

From the practical standpoint, our data (and the data on Factor V) imply as Quick has frequently reiterated (see Ref 20), that a 1½ to 3-second prolongation of the prothrombin time should not be shrugged off automatically as a vagary of the one-stage provided that it occurs consistently and that the control is reproducible from day to-day, we would add that a similar occurrence with the thromboplastin generation test may have a similar significance. The observer may be dealing in either instance with an individual heterozygous for an abnormal gene affecting one or another of the prothrombin accessory factors The obvious conclusion is that all such plasmas should be assayed specifically for the possible deficiencies

The fact that the heterozygotes in the Stuart kindred did not consider themselves symptomatic does not imply that this is always true. We have recently studied another kindred reputed to be female bleeders We discovered that roughly half the females examined had 1 to 3-second prolongations of plasma prothrombin time and 40 to 60 per cent Stuart factor levels as measured by the assays used in the present communication These women were known to be bad operative risks by their physicians and to be persistently anemic, presumably because of menorrhagia since their anemia improved during pregnancy and after menopause. The frequency of the mild disorder in the family and the intermediate Stuart factor levels led us to conclude that the patients were heterozygous

If the three patients of de Vries Kettenborg and van der Pol (9) with abnormal thromboplastin generation tests had Stuart factor deficiency they were probably heterozygous also judging from their prothrombin times It is of interest that all three of these patients were males Their presenting symptoms, gastrointestinal bleeding are not surprising in view of the well-known excess (4 1) of gastro-duodenal ulcer in males. Nevertheless because of menstruation in women and the increased frequency of operations it seems likely that more women than men heterozygous for the autosomally transmitted clotting dyscrasias will be referred to the laboratory as doubtful bleeders

One member of the Stuart kindred III 5 created a classification problem However he was scored as normal since his Stuart factor level (86 per cent) was more than 3 standard deviations greater than the mean of the indubitable heterozygotes This conclusion could have been tested further by examining his 13 children as half of them would be expected to be heterozygous under our hypothesis if the father were in fact heterozygous These studies were not carried out primarily because whether or not he is heterozygous does not affect the main hypothesis Also since he lives 200 miles from our laboratory in a remote mountain area, testing all his children in the field constitutes a separate enterprise However study of his descendants is intended eventually since it is of more than passing interest to discover whether heterozygosity for the Stuart defect can be present in a person with a normal level of Stuart factor We have also not yet attempted to study the turnover rate of the Stuart factor in the proband or the steady state relationships in the heterozygotes It is anticipated that these studies will be performed in the future.

A rough calculation of the frequency of the abnormal Stuart gene will be made because it emphasizes our point about consistently prolonged prothrombin times We are aware of only one living homozygote for Stuart factor deficiency in North Carolina in a population of somewhat more than 4 000 000 a frequency of about 00000025 However Lewis Fresh, and Ferguson (2) studied another now deceased and Crockett and associates (4) studied an identical patient in Virginia If this frequency figure is quadrupled to compensate for incomplete ascertainment, it should give a *probable upper limit* of the frequency (000001) Then if the Hardy-Weinberg conditions are assumed to apply the upper limit of the gene frequency can be estimated efficiently as the square root of the frequency of the homozygous class or

$$q = \sqrt{000001} = 001$$

Then the frequency of the normal allele can be obtained as $p = (1\text{-}q)$ or $(1\ 001) = 999$ and the

frequency of heterozygotes as

$$2pq = 2(.999)(.001) = .0019$$

or about 2/1,000. This surprisingly high heterozygote frequency implies that if one surveyed a random population of North Carolinians by the prothrombin time test he might encounter as many as two persons in each thousand with prothrombin time consistently prolonged 1½ to 3 seconds where the prolongation resulted from heterozygosity for Stuart factor deficiency.

Finally, the demonstration that the abnormal autosomal gene for Stuart factor deficiency is incompletely recessive implies that the normal allele is incompletely dominant. This means biochemically either that a single normal allele at the Stuart locus does not furnish enough enzymatic activity or substrate at some point in the synthetic metabolic pathway to maintain the level of the factor at that of "wild type," or that the mutant gene inhibits some step (21). The normal allele at this locus is therefore, in its overall effect, like that for Factor V in some pedigrees (18, 19), some pedigrees of Christmas disease (22, 23), and some pedigrees of mild hemophilia (24), and unlike the allele for classic hemophilia (22, 23, 25).

SUMMARY

1. Stuart factor deficiency, recently segregated from the heterogeneous group of hemorrhagic states known variously as SPCA, proconvertin or Factor VII deficiency, has been studied in a large North Carolina kindred and shown to be inherited as a highly penetrant but incompletely recessive autosomal characteristic.

2. The heterozygotes have been found to be only mildly affected or normal clinically but to have 1½ to 3-second prolongations of the prothrombin time and thromboplastin generation tests. These effects are, presumably, due to the reduction in the level of this factor to a mean of 36 per cent in the range 20 to 52 per cent.

3. It is pointed out that carrier detection may be possible in many instances with fairly simple tests.

4. It is emphasized that heterozygotes for the various hemorrhagic states, especially females, may be encountered as patients with abnormal operative bleeding, persistent gastro-intestinal bleeding or menorrhagia with persistent anemia. Such symptoms, in the absence of clean-cut laboratory evidence, however, should not be assumed to represent heterozygosity.

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AGE DIFFERENCES IN THE INTRAVENOUS GLUCOSE TOLERANCE TESTS AND THE RESPONSE TO INSULIN

By FELIX A SILVERSTONE,[1] MARTIN BRANDFONBRENER,[2] NATHAN W SHOCK, AND MARVIN J YIENGST

(From the Gerontology Branch, National Heart Institute, National Institutes of Health, Bethesda, and the Baltimore City Hospitals, Baltimore, Md)

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In addition to providing information with regard to the status of carbohydrate metabolism, the time course of the disappearance of injected glucose from the blood stream offers information concerning the overall effectiveness of a variety of physiological mechanisms involved in maintaining homeostasis In a number of investigations, reduction in glucose tolerance, *i e*, a slower rate of return to fasting levels of blood sugar following the oral (1–17) or intravenous (18–20) administration of glucose, has been reported in older people The diminished glucose tolerance in the older individual might be due to (a) inadequate release of insulin from the pancreas, or greater inactivation of endogenously released insulin, (b) the loss of functioning protoplasm with increasing age so that less metabolizing tissue is removing glucose from the blood, (c) a diminution in the effectiveness of the metabolic processes involved in the removal of sugar from the blood stream, (d) alterations in the rate of release of glucose from the liver, or (e) a reduction in the volume in which the glucose is originally distributed in the aged By comparing the glucose and glucose-insulin tolerance tests in the same individual, an estimate of the effect of insulin may be obtained (21–27) In the experiments to be reported standard amounts of insulin were administered along with glucose to both old and young subjects with the aim of investigating age differences in the response to the insulin

EXPERIMENTAL METHODS

Subject selection Thirty-five male subjects, age 23 to 86 years, were selected on the basis of a detailed history, physical examination and a series of laboratory tests The presence of any of the following served to exclude a subject from the study (a) history or known evidence of diabetes or glycosuria (b) severe alcoholism, hepatomegaly, cirrhosis or other liver disease, (c) cardiac decompensation or edema, (d) infections, temperature elevation or acute or chronic trauma (including surgical) within one week of test, or (e) the taking of steroid drugs (other medication such as aspirin was omitted for 12 hours preceding the tests) All were ambulatory in-patients on a routine full hospital diet for at least one week Fasting blood sugars were within normal limits (27, 28) as shown in Table I

Experimental procedure The intravenous glucose tolerance test (GTT) and the glucose insulin tolerance test (GITT) were performed in each subject under basal conditions and separated by an interval of not less than one week In 8 of the subjects, each of the tests (GTT and GITT) was carried out twice in order to evaluate reliability

Twenty minutes before either test was begun, a modified Lindeman needle was placed in an antecubital vein and left in place for the duration of the test The needle was kept patent by heparinization of the stylus and was used subsequently only for withdrawing blood specimens without a tourniquet (29) A vein in the opposite arm was used for the injection of 50 ml of 50 per cent glucose in water over a period of two minutes For the GITT, 5 units of hyperglycemic factor-free insulin[3] (Lilly), per square meter of body surface area, were rapidly injected, followed immediately by the standard amount of glucose The fasting blood specimen was obtained through the Lindeman needle a few minutes before zero time, which was recorded as the beginning of the injection of glucose

Blood samples were collected at 5-minute intervals for the first hour and at 20 minute intervals during the second hour, and were placed immediately in tubes containing a dried heparin and sodium fluoride mixture All analyses were completed the day of the test, using the Nelson-Somogyi method (30) Determinations were in duplicate and were read on a Model DU Beckman Spectrophotometer

Data analysis For each tolerance test, the observations obtained between 10 and 60 minutes of the experiment were fitted to the equation $\log_e y = \log_e A - kt$ ($y = Ae^{-kt}$) where y is the blood glucose concentration in

[1] Present address Maimonides Hospital, Brooklyn, New York

[2] Present address Presbyterian Hospital, New York, New York

[3] We are indebted to Dr W R Kirtley of Eli Lilly and Company for supplying us with HGF free Z I C 40 units per ml

TABLE I

Subject characteristics

Variable		Age group: Young	Middle	Old	Total
N		12	11	12	35
Age (*yrs*)	Mean	31.3	49.1	78.7	53
	Range	23	42	65	23
		37	58	87	87
Height (*cm*)	Mean	175.3	174.4	162.6	170.8
	σ_{Mean}	2.0	2.7	2.3	2.2
	Range	165.7	165.1	143.5	143.5
		185.4	186.7	171.4	186.7
Weight (*Kg*)	Mean	71.0	64.6	66.8	67.5
	σ_{Mean}	2.4	3.5	3.9	3.2
	Range	57.3	52.0	45.1	45.1
		85.0	92.7	88.2	92.7
Surface area (M^2)	Mean	1.83	1.77	1.70	1.77
	σ_{Mean}	.033	.047	.060	.040
	Range	1.67	1.58	1.39	1.39
		2.00	2.08	2.00	2.08
Fasting blood glucose*	Mean	68.0	78.5	85.0	77.1
	σ_{Mean}	1.9	2.0	2.3	2.1
	Range	60	64	70	60
		77	90	97	97
Glucose administered (*Gm./Kg body wt.*)	Mean	.356	.397	.389	.381
	σ_{Mean}	.012	.018	.024	.020
	Range	.294	.270	.283	.270
		.436	.454	.554	.554

* Mean of control observations on glucose and glucose-insulin tests.

milligrams per 100 ml. and t is time in minutes, following the injection of the glucose load. The method of least squares (31) was utilized for the computation of A and k for each experiment. The value of k was taken as the index of tolerance for this study. The difference between the k for the glucose (k_G) and the glucose-insulin test (k_{GI}) is called Δk and served as the index of the response to insulin in each subject. Visual fits to plots of log of the glucose level against time were also made and compared with the least squares fitting.

Age changes in the data were evaluated by determining the regression of the derived measures on age and also by comparing mean values for three groups: young (12 subjects, age 20 to 39 years), middle (11 subjects, age 40 to 59 years), and old (12 subjects, age 60 to 90 years).

RESULTS

Characterization of the subjects

No significant ($P = > 0.10$) differences were found among the three age groups with respect to body weight, surface area or dose of glucose per Kg. of body weight (Table I). A small but statistically significant increase in fasting venous blood sugar levels with age was observed in this sample.

Reliability of methods

The standard deviation of repeated glucose determinations on a single filtrate was ± 1.1 mg. per cent ($N = 58$). Comparing two filtrates, prepared from the same blood sample, the standard deviation was 2.1 mg. per cent ($N = 53$).

There was no systematic difference between fasting blood sugar levels determined on the same individual on different days. The standard error of estimate between measurements made on the first and second days was ± 6.5 mg. per 100 ml.[4]

In the 8 subjects (4 old and 4 middle-aged) who had duplicate glucose tolerance and glucose-insulin tolerance tests, no significant differences occurred between the results of the first and second tests with respect to k_G or k_{GI}. The dupli-

[4] Mean values are reported with standard errors of the mean.

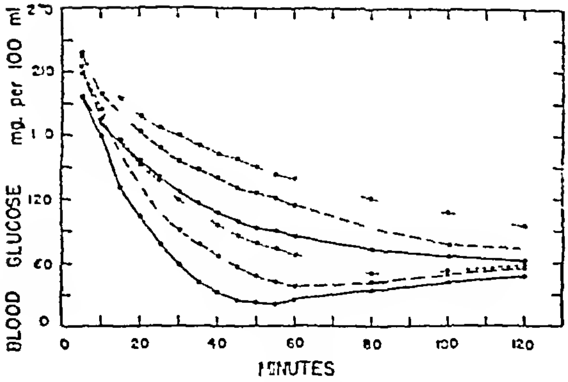

FIG 1 LINEAR PLOTS OF AVERAGE BLOOD SUGAR VALUES (MG PER 100 ML) AT EACH TIME INTERVAL (MIN) FOLLOWING THE INTRAVENOUS ADMINISTRATION OF 25 GM GLUCOSE FOR EACH OF THREE AGE GROUPS

Glucose tolerance (GTT) open circles, glucose plus 5 units insulin per sq M surface area (GITT) closed circles old subjects (65 to 87 years old) dotted lines middle aged subjects (42 to 58 years old) dashed lines, and young subjects (23 to 37 years old) solid lines

cate tests were not performed in any specific order, the lapse of time between tests varied from one week to three months In contrast to results reported by Hlad, Elrick, and Witten (32) values for k_G or k_{GI} were repeatable and characteristic for the individual

General description of results

Average values of blood sugar concentration at each time interval, following the administration of glucose, are plotted for each of the three age groups in Figure 1 The rate of fall in blood glucose level was greater for the young than for the old subjects under both experimental conditions When tests of significance of age differences were applied to specific time points along the glucose or glucose-insulin tolerance curves, true differences ($P < 0.001$) were found between young and old subjects at 15, 30, and 60 minutes after injection of the glucose Differences over shorter age spans, *i e*, between young and middle, and middle and old subjects, were usually significant at $P < 0.01$ or $P < 0.05$

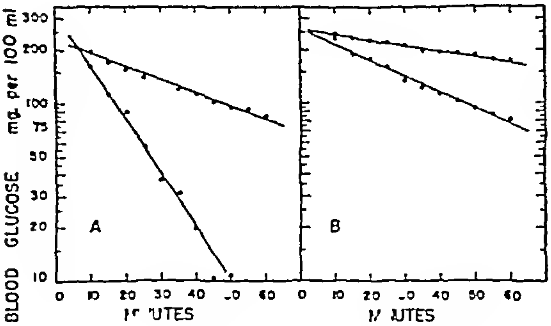

FIG. 2. ESTIMATION OF K_G AND K_{GI} IN INDIVIDUAL EXPERIMENTS

Log glucose concentration in blood (mg per 100 ml) plotted against time (min.) A. Young subject (26-year-old) B Old subject (86-year-old) Upper lines (open circles) represent data from GTT, lower lines (solid circles) represent data from GITT

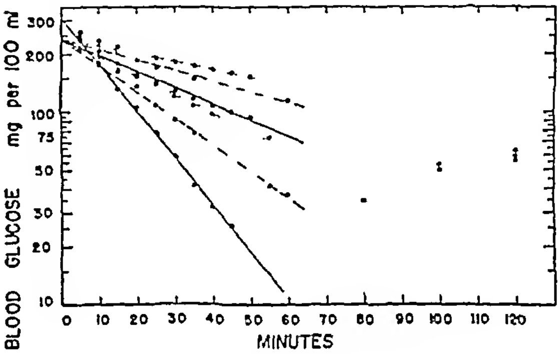

FIG 3 AGE DIFFERENCE IN K_G AND K_{GI}

Plots of log glucose concentration in the blood (mg per 100 ml) against time (min) following intravenous administration of 25 Gm. glucose. Glucose tolerance (GTT) open circles, glucose plus 5 units insulin per M² surface area (GITT) solid circles, old subjects (65 to 87 years old) dotted lines, middle aged subjects (42 to 58 years old) dashed lines, and young subjects (23 to 37 years old) solid lines (Lines are fitted to points for 10 to 60 min inclusive.)

Age differences in rate of disappearance of glucose from the blood

When the log glucose concentration was plotted against time, a linear relationship was obtained for the points between ten and 50 to 60 minutes Sample plots for a young (26-year-old) and an old (86-year-old) subject are shown in Figure 2A and 2B Figure 3 shows the log of the mean glucose values for the three groups of 8 subjects, plotted against time after glucose administration Deviations from linearity are apparent in all curves beyond 50 or 60 minutes Therefore, the expression $\log_e y = \log_e A - kt$ fails to describe the total process, but may be used to derive an index of the rate of disappearance of glucose from the

Subject	Condition	Age															80	100	120	k (%/min.)
J B	G	3															68	65	67	1.73
	GI																			8.26
F B	G	3															60	63	73	3.13
	GI																39	42	51	5.30
E C	G	3															66	55	52	1.60
	GI																			9.36
L. C	G	3															83	75	67	1,39
	GI																31	55	60	3.92
R H	G	3															68	51	41	1.55
	GI				67	194	142	112	83	55	40	27	13	7	2	6	27	33	40	9.30
J J	G	27	60.4	172.7	67	216	190	170	158	152	147	139	132	115	116	108	84	70	60	1.36
	GI				69	256	159	139	110	88	67	49	42	37	27	30	43	46	57	4.74
D L	G	37	71.0	176.5	60	176	166	128	107	88	68	73	71	60	68	59	56	68	59	2.81
	GI				73	142	113	82	59	38	17	17	17	23	29	29				7.80
M N	G	37	83.4	175.3	74	193	176	167	152	141	135	126	119	112	109	102	83	72	65	1.26
	GI				68	160	117	94	74	54	42	37	22	23	23	31	37	42	49	5.07
T R	G	23	57,3	171.4	65	191	169	156	135	128	119	113	102	93	85	80	58	52	49	1.70
	GI				55	153	120	91	74	52	30	25	12	12	16	18	22	28	35	6.88
B T	G	28	72.2	185.4	66	195	172	155	139	127	120	112	102	94	92	84	66	58	53	1.63
	GI				60	161	112	89	57	37	31	19	9	10	17	19	23	27	27	7.78
C W	G	25	61.8	168.9	68	202	185	186	172	161	149	137	124	120	118	110	91	73	69	1,27
	GI				73	221	162	142	105	91	76	51	48	39	35	33	50	51	59	3.85
J Y	C	31	85.0	184.2	73	201	185	183	176	170	165	155	147	142	139	136	123	109	97	0.78
	GI				73	175	136	93	74	55	45	37	35	29	22	21	33			4.44
Mn	G	31	71.0	175,3	67	191	171	156	141	127	116	107	99	93	90	85	73	66	62	1.68
	GI				69	179	131	103	77	58	41	32	25	22	21	25	34	41	47	6,39
σ_M	C		2,4	2.0	2.1		5.8			9.7			10.7			8.9				19
	GI				2.0		6.1			3.8			4.3			2,3				60

TABLE III

Blood glucose levels at specified times following intravenous administration of 25 grams glucose (G) and intravenous administration of 25 grams glucose plus 5 units per M^2 of insulin (GI) in middle aged males

Subject	Condition	Age (yrs)	Wgt (kg)	Ht (cm)	Blood sugar levels (mg /100 ml at specified time—min) 0	10	15	20	25	30	35	40	45	50	55	60	80	100	120	k (%/min)
S C	G	12	65 8	165 1	78	231	209	199	184	179	164	155	114	140	131	124	103	86	73	1 21
	GI				81	216	198	171	144	126	111	96	87	77	68	65	51	51	59	2 51
C C	G	53	66 9	182 9	73	199	157	126	107	86	73	71	59	65	69	62	56	58	69	3 11
	GI				78	159	121	96	71	57	46	36	35	28	20	21				1 39
W D	G	43	61 8	186 7	92	231	215	189	170	148	149	139	130	121	115	106	73	73	76	1 19
	GI				87	213	167	136	105	93	71	63	50	16	40	37	11	50	56	3 56
J D	G	42	72 5	166 4	77	216	200	187	172	164	149	143	135	131	126	117	100	89	75	1 20
	GI				73	205	167	133	102	86	71	59	46	40	29	31	11	39	50	1 21
A E	G	58	60 9	185 4	76	224	198	185	174	167	159	158	145	138	136	128	112	96	81	1 02
	GI				85	214	192	173	146	126	107	96	81	69	58	53	39	52	45	2 91
L G	G	50	55 7	167 6	66	218	197	173	162	145	134	127	114	103	96	91	62	58	47	1 72
	GI				62	169	138	116	96	75	59	45	35	25	26	15	26	41	19	1 71
E H	G	54	55 7	170 2	70	231	226	212	208	198	187	181	170	168	157	150	129	111	86	0 87
	GI				85	199	170	128	106	98	81	71	61	46	40	40	43	51	54	3 48
D J	G	58	52 0	166 4	80	248	216	190	174	158	152	148	144	138	128	121	97	80	75	1 29
	GI				80	206	180	157	139	113	109	100	92	80	75	69	44	55	59	2 17
J J	G	44	92 7	175 3	71	193	175	167	150	139	137	128	119	115	110	106	89	80	74	1 19
	GI				86	180	142	121	91	69	74	60	48	42	32	32	59	67	72	3 49
J R	G	53	71 1	185 4	87	220	186	175	163	149	141	135	124	123	116	109	93	88	83	1 29
	GI					193	153	119	93	77	63	56	47	44	34	33	32	45	57	3 55
R W	G	13	55 1	167 6	80	249	227	214	193	188	181	166	162	152	146	138	106	87	79	1 13
	GI				70	195	147	118	77	70	55	49	37	28	25	23	39	43	49	4 67
Mn	G	49	64 6	174 4	77	223	201	183	169	156	148	141	131	127	121	114	93	78	74	1 41
	GI				79	196	162	134	107	90	77	67	56	48	40	37	42	49	55	3 61
SE_{Mn}	G		3 5	2 7	2 3		6 5			9 0			8 9			6 9			3 1	21
	GI				2 6		8 1			7 2			6 4			5 8			2 4	25

TABLE IV

Blood glucose levels at specified times following intravenous administration of 25 grams glucose (G) and intravenous administration of 25 grams glucose plus 5 units per M^2 of insulin (GI) in aged males

Subject	Condition	Age (yrs.)	Wgt. (Kg)	Ht. (cm.)	Blood sugar levels (mg./100 ml. at specified time—min.) 0	10	15	20	25	30	35	40	45	50	55	60	80	100	120	k (%/min)
F B	G	72	68.2	143.5	80	223	195	181	162	150	144	132	127	121	116	109	94	86	77	1.36
	GI				88	193	162	143	132	120	105	99	89	77	74	68	46	51	58	2.04
T B	G	86	56.1	160.0	90	243	219	204	195	178	168	158	150	139	133	130	105	95	79	1.26
	GI				74	182	151	142	117	107	100	84	78	64	57	59	39	41	42	3.58
J H	G	73	53.5	161.3	75	211	194	180	176	171	162	155	152	148	146	158	108	108	95	0.79
	GI				65	188	150	134	120	94	83	76	65	56	51	49	39	37	42	2.74
E K.	G	76	55.6	156.8	83	238	238	217	205	204	197	181	166	165	157	152	136	120	105	0.96
	GI				80	232	208	184	165	150	138	124	118	112	99	85	67	54	57	1.88
J He	G	85	70.1	162.6	95	255	223	190	184	186	181	178	174	165	155	141	142	131	114	0.91
	GI				83	202	170	153	135	121	98	86	71	69	66	60	54	63	71	2.52
W L.	G	87	83.4	168.9	92	220	204	184	177	167	156	155	144	141	134	130	113	102	88	0.82
	GI				96	192	152	128	111	95	74	76	62	59	64	51	45	53	71	2.95
D M	G	84	45.1	156.2	82	278	258	236	211	206	188	182	174	163	141	141	122	100	88	1.35
	GI				73	223	193	160	142	119	108	89	75	65	54	50	36	33	39	3.06
J M	G	65	82.4	171.4	96	220	216	211	205	194	189	185	179	176	165	155	147	122	104	0.67
	GI				94	212	197	180	168	148	141	130	118	106	103	93	69	63	66	1.66
G P	G	79	60.7	171.4	86	206	194	179	176	171	166	162	158	151	149	143	127	113	101	0.67
	GI				86	186	177	154	143	131	121	109	101	94	86	80	58	49	58	1.73
D R.	G	81	61.8	161.3	78	211	193	174	164	149	135	132	124	113	111	105	92	80	71	1.36
	GI				78	210	171	140	118	97	67	64	54	51	38	31				3.74
A T	G	73	77.0	167.6	101	247	232	228	209	212	198	191	192	186	176	174	149	136	123	0.68
	GI				92	231	190	168	159	135	122	113	103	94	86	81	66	66	74	2.04
L. W	G	82	88.2	1,0 2	83	215	201	186	183	173	165	162	150	144	138	138	119	101	99	0.91
	GI				83	201	174	144	140	128	121	101	91	89	97	89	73	68	64	1.99
Mn	G	78	66.8	162.6	87	231	214	198	187	180	171	164	158	151	143	140	121	108	95	0.98
	GI				83	201	175	152	138	119	107	96	86	78	73	67	50	53	58	2.49
SE_{Mn}	G		3.9	2.3	2.3		6.0			6.0			5.9			5.7				.08
	GI				2.7		5.6			5.4			6.2			5.2				.02

TABLE V

Rate of disappearance of glucose from the blood with (k_{GI}) and without insulin (k_G)
(k expressed as per cent per minute)

		Venous blood							Arterial blood*	
		Glucose (k_G)		Glucose-Insulin (k_{GI})		Diff (Δk)			Glucose (k_G)	
Age group	N	Mn.	σ_{M_n}	Mn.	σ_{M_n}	Mn.	σ_{M_n}	N	Mn	σ_{M_n}
Young	12	1 68	19	6 39	60	4 12	59	13	1 94	20
Middle	11	1 44	21	3 61	25	2 17	26	13	1 61	09
Old	12	0 98	08	2 49	02	1 52	17	38	1 28	06
Total	35	1 37	11	4 15	11	2 62	09	64	1 48	07

* Data from Smith and Shock (19) recalculated

blood during the first 50 to 60 minutes of the experiment Examination of Figure 3 shows that (a) the early rate of disappearance of excess of glucose from the blood was more rapid in the young than the old, (b) the rate of disappearance was increased in all age groups by the simultaneous administration of insulin, and (c) the influence of insulin was greater in the young than the old subjects

Computations by the method of least squares of the slope for each individual test provided data for estimates of the variability of k within each age group Tables II, III, and IV present the values of k for both the GTT and the GITT with increasing age Average values of k, expressed as per cent per minute, for the young and old, respectively, were as follows k_G, 1 68 and 0.98, k_{GI}, 6 39 and 2 49, and Δk ($k_{GI} - k_G$), 4 12 and 1 52 Values of *P* were less than 0 01 for these age differences Regressions of k on age were significant at the 0 01 level for k_G and at less than 0 001 for k_{GI} and Δk The effect of insulin in the average adult male was to increase the rate of fall in blood glucose level from 1 37 per cent per minute to 4 15 per cent per minute

DISCUSSION

Subject selection—Activity and diet

Since age differences in glucose tolerance are relatively small, it is necessary to give careful consideration to the selection of subjects In order to minimize the effects of prior diet, which has been shown to have an influence on the glucose tolerance (33–36), only subjects who had been on a standard hospital diet for at least one week were tested in the present series

Since reduced activity has been shown to reduce glucose tolerance (36, 37), only ambulatory patients were studied The young subjects were also patients drawn from an ambulatory in-hospital population Thus, the level of activity was probably more uniform between the different age groups than would have been the case had staff members been used for the younger age groups

Dose of glucose and insulin

Although different amounts of glucose have been used by previous investigators, recent studies have shown that adjustment of the dose of glucose to body size is unnecessary (18, 28, 38) Consequently, a standard dose of 25 Gm of glucose was administered at a uniform rate (18, 38) to all subjects in the present study The glucose load varied from 0 57 Gm per Kg to 0 27 Gm per Kg in different subjects, but there were no systematic differences between age groups (Table I) The dose of insulin was set at 5 units per M^2, which is approximately 0 1 unit per Kg body weight Experimental studies (22–24, 27, 39–41) indicate that insulin gives a maximum effect on rate response at dose levels of 0 05 unit per Kg and up to 0 3 unit per Kg The effect of differences in endogenous insulin production would be obliterated at this dose range of injected insulin

Comparison of venous and arterial blood samples

Nelson's modification of the Somogyi method (30) was used for sugar determination in this study in order to minimize the effects of nonfermentable reducing substances (42, 43) The use of venous blood samples in conjunction with an

indwelling needle has the advantages of good patient acceptability and a minimum of patient trauma (44–46). Although the differences between arterial and venous blood glucose levels are small under fasting conditions (arterial bloods average 9 mg. per 100 ml. higher than venous) there is a wide range of individual variation (1 to 17 mg. per 100 ml. in normal subjects) and the difference increases markedly (average 30 to 43 mg. per 100 ml.) following the administration of glucose (43, 47). Since Blotner (37) has found that glucose tolerance determined on venous blood samples was influenced less by physical activity in both children and adults than were estimates derived from capillary blood samples it follows that the use of venous blood might be a more adequate test of age differences in glucose tolerance.

Since previous studies of glucose tolerance from this laboratory were based on arterial blood samples (19) the data were fitted to the tolerance equation. A total of 64 subjects divided into three age groups were tested under conditions closely approximating the present study except that blood samples were drawn from the femoral artery (19). Insulin was not given. Table V gives the k values based on arterial blood samples.[5] As was true for venous blood samples there was a significant decrement in k_0 with increasing age. However the trend toward increasing fasting blood sugar levels with age found in the present study on venous blood and in the report on capillary blood by Schneeberg and Finestone (20) was not apparent in arterial blood.

The tolerance equation

One simple expression which can serve to express the rate of disappearance of glucose from the blood, as a single number is the equation $\log_e y = \log_e A - kt$ (48–51). If one assumes that no distinction is made between the glucose added to the blood from an external source and the glucose added by the liver or other cells of the body the estimates of the slope of the curve must be made on the total glucose content at successive time intervals. This assumption may be applied safely only to the early parts of the curve since it is obvious that alterations in the glucose concentration will be introduced by other processes in the body which tend to add glucose to the circulation particularly when blood sugar levels fall to low values as in the case when insulin is administered. Greville (50) as well as Hlad, Elrick and Witten (32) found that subtraction of a calculated asymptotic value of blood sugar level resulted in a somewhat better fit to glucose tolerance data beyond 90 minutes. In this study where curves were limited to the first 60 minutes the fit was very good, the introduction of an asymptote had only a small insignificant effect on goodness of fit. Furthermore the use of an asymptote gave rise to difficulties in comparing the GTT and the GITT since subtraction of a calculated asymptote for the glucose-insulin curves often resulted in values less than zero. Although the value of k is related to the level of the asymptote, both parameters are determined by the same set of experimental points.

In agreement with other studies (49–51) the blood glucose level at 5 minutes following the injection was found to be higher than predicted from the exponential curve suggesting an interaction with the early extra-cellular mixing phase. By 10 minutes after the glucose injection the mixing phase is indistinguishable from the body of the curve. Conard, Franckson, Bastenie, Kestens and Kovacs (52) injected glucose and thiocyanate simultaneously and found a thiocyanate space of 14.31 L. and a glucose space of 14.01 L. In our experiments the average glucose space was 14.1 L. or 22 per cent of the body weight. This value is only slightly lower than the glucose space (23.3 per cent of body weight) reported by Hlad, Elrick and Witten (32) on the basis of continuous infusions of glucose. Since there are no significant changes with age in the thiocyanate space (53) it appears that the values of k are determined by the distribution of glucose in

[5] Arterial k values were based on a visual fit. A comparison between the derivations of k by least squares and graphic estimates made from a visually fitted line using the venous data, gave mean values of 137 ($k \times 10^4$) for both methods for the GTT ($r = 0.96$). For the GITT the mean values of k by the least squares method was 415 as compared to 439 by the visual method ($r = 0.89$). Age did not influence the correlation between methods. Thus a visual fit to the data yields substantially the same results as analysis by least squares. However the latter permits a quantitative statement of the "goodness of fit" of the equation. This is not possible when the visual method above is used. Age did not influence the goodness of fit.

TABLE V

Rate of disappearance of glucose from the blood with (k_{GI}) and without insulin (k_G)
(k expressed as per cent per minute)

		Venous blood							Arterial blood*	
		Glucose (k_G)		Glucose-insulin (k_{GI})		Diff (Δk)			Glucose (k_G)	
Age group	N	Mn.	σ_{M}	Mn	σ_{M}	Mn	σ_{M}	N	Mn.	σ_{M}
Young	12	1.68	19	6.39	60	4.12	59	13	1.94	20
Middle	11	1.44	21	3.61	25	2.17	26	13	1.61	09
Old	12	0.98	08	2.49	02	1.52	17	38	1.28	06
Total	35	1.37	11	4.15	11	2.62	09	64	1.48	07

* Data from Smith and Shock (19) recalculated

blood during the first 50 to 60 minutes of the experiment. Examination of Figure 3 shows that (a) the early rate of disappearance of excess of glucose from the blood was more rapid in the young than the old, (b) the rate of disappearance was increased in all age groups by the simultaneous administration of insulin, and (c) the influence of insulin was greater in the young than the old subjects.

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Milton Landowne for his thoughtful criticism Dr Max Halperin for statistical consultation Miss Janet Lewis and Mr William Martin for technical assistance, and to Mrs. Elizabeth Benser for computational aid.

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the intra-cellular fluids and metabolic pathways and are influenced very little by extra-cellular mixing and not in a manner which is age biased

Age differences

Although there is an overall reduction in the rate of removal of glucose from the blood and a reduced response to insulin with increasing age, it cannot be assumed that this reduction is necessarily associated with altered cellular metabolism A similar overall effect could result from a reduction in the number of metabolizing units Other studies from this laboratory have indicated a gradual loss of metabolizing tissue with increasing age (54) This conclusion is based on the observed decrease in intra-cellular water (antipyrine space minus thiocyanate space) with age The intra-cellular space, calculated in this fashion, averages 10 5 L in young and 8 6 L in aged subjects, a reduction of 18 per cent Over the same age span, k shows a reduction of 42 per cent for the glucose tolerance data and 61 per cent for the glucose-insulin tests Although the data do not represent observations made on the same subjects, and the dimensions are incongruous, it seems difficult to account for all of the changes observed on the basis of a loss of functioning protoplasm alone

It is conceivable that the age differences in the rate of removal of glucose from the blood might be due to differences in blood flow and delivery of glucose to the tissues Clearly, a reduction in the total amount of blood delivered, per unit of time, would influence k if all the glucose were removed in a single passage through a vascular bed However this is not the case If there were a substantial reduction in blood flow to tissues in the older subject, an increase in the A-V difference should appear Since we do not have simultaneous arterial and venous glucose levels on the same subjects, no final decision can be reached on this question, but it does not seem likely that differences in blood flow can account for the age differences observed

It is recognized that the concentration of glucose in the blood at any given time represents an equilibrium between the rate of removal and the rate of release of glucose from the liver The differential effect of insulin in the three age groups makes it improbable that the results obtained can be ascribed to differences in the rate of release of glucose from the liver in the old and young subjects Recent studies indicate that the early effect of insulin action is that of increasing peripheral uptake and metabolism of glucose The liver response is minimal and delayed (55)

Inactivation of insulin by a plasma constituent may be a factor in some phases of diabetes, particularly in regard to the mechanism of clinical insulin resistance Welsh, Henley, Williams, and Cox (56) studied the plasma binding of insulin I^{131} in 118 subjects, 43 of which were non-diabetic ranging from 14 years to 90 years of age Analyzing their data with reservation for the inclusion of patients with other active disease in their group of non-diabetic controls, no trend is discernible between the age of the subject and the potential insulin inactivation by plasma binding

Although proof cannot be offered, it seems reasonable to assume that at least part of the age differences can be ascribed to alterations in the metabolic effectiveness and response of the functioning cells in the aged male

SUMMARY

Intravenous glucose tolerance and glucose-insulin tolerance tests were performed on 35 normal male subjects under standardized conditions using venous blood samples The subjects ranged in age from 23 to 86 years Blood samples drawn at 5-minute intervals, between 5 and 60 minutes after administration of 25 Gm glucose, were analyzed for glucose by the Nelson method The rate of fall of the blood sugar level between 10 and 60 minutes was determined by fitting the experimental points to the equation $\log_e y = \log_e A - kt$ A significant decrease in k with age was observed in both the glucose and the glucose-insulin tolerance curves The administration of insulin had a greater effect on the rate of disappearance of glucose from the blood in the young than the old subjects It is proposed that the age difference may result from both a reduction in the amount of functioning protoplasm and an alteration in intra-cellular glucose metabolism

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THE RENAL RESPONSE IN MAN TO ACUTE EXPERIMENTAL RESPIRATORY ALKALOSIS AND ACIDOSIS[1]

By E. S. BARKER,[2,3] R. B. SINGER,[4] J. R. ELKINTON[2] AND J. K. CLARK

(From the Renal Section and Chemical Section of the Department of Medicine The Department of Research Medicine and the Department of Biochemistry the University of Pennsylvania School of Medicine Philadelphia Pa.)

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The experimental results to be presented here deal with the renal component of the multiple effects in man of acute experimental respiratory alkalosis (hyperventilation) and acidosis (CO_2 inhalation) One aim of the experiments has been to define an integrated picture of the total body response to acute respiratory acid base disturbances A previous paper (1) contained a description of the effects observed in the same experiments on the composition of plasma and red cells and a quantitative estimate of the exchanges of ions and water between red cells plasma, extracellular fluids and a phase or phases outside the chloride space ('intracellular') In the present report consideration is given to the changes in renal excretion and hemodynamics and an attempt is made to define more clearly certain mechanisms involved Reference should be made to the previous report (1) for data on the blood or plasma changes only a few of these data are included here when they are directly important to interpretation and renal effects The present findings were previously reported in abstract (2 3)

EXPERIMENTAL PROCEDURE AND CHEMICAL METHODS

Six normal male subjects actively hyperventilated and six inhaled CO_2 as described in detail in the preceding paper (1) Following control periods of 47 to 74 minutes duration, hyperventilation was carried out for approximately 30 minutes in 5 of the 6 experiments, and for twice that period in the last experiment 7.5 to 7.7 per cent CO_2 in air or oxygen was inhaled for 21 to 30 minutes. Measurements were continued in both types of experiments during subsequent recovery periods which ended 97 to 145 minutes after onset of the stimulus (designated time zero) Standard water loading was carried out before the experiments and continued throughout with water given in amounts equivalent to urine excreted. In respiratory acidosis a neutral or slightly alkaline control urine was considered desirable to facilitate observation of renal effects. Accordingly in experiments 1 to 5 inclusive, the subjects were given 4.2 gm. $NaHCO_3$ (50 mEq.) by mouth at −95 to −150 minutes. As a control in the sixth experiment NaCl was substituted for the $NaHCO_3$ and this experiment is not included in statistical analyses. Four additional control experiments were done in which the $NaHCO_3$ load was given, and observations were made for the usual experimental time (but with no respiratory stimulus) to indicate the effect of the loading procedure alone.

Renal clearances were determined by standard techniques and chemical methods (4 5) with bladder catheterization and appropriate anaerobic collection of urine to prevent loss of CO_2. Changes in glomerular filtration rate were estimated from changes in endogenous creatinine clearance and effective renal plasma flow from p-aminohippurate (PAH) clearance.[5] Following an equilibration period of at least 45 minutes urine was collected for 3 periods before, 1 to 4 periods during and 3 to 4 periods after the application of the stimulus (hyperventilation or CO_2 inhalation)

Total CO_2 was determined in the anaerobically collected urine by the manometric method of Van Slyke and Sendroy (6) urine pH at 37 C by means of the photocolorimetric method of Van Slyke, Weisiger and Van Slyke (7) sodium and potassium with a Barclay internal standard flame photometer (8) chloride by the

[1] Laboratory facilities were aided by grants from the National Heart Institute of the United States Public Health Service (Grants H-405 and H-340) the Life Insurance Medical Research Fund, and the C. Mahlon Kline Fund for Development in the Department of Medicine.

[2] Established Investigator of the American Heart Association.

[3] In part during tenure of Post-doctorate Fellowship of the National Institutes of Health, United States Public Health Service.

[4] Present address 501 Boylston Street, Boston, Mass.

[5] Excretion of anionic PAH^- in itself has some effect on urinary acid base pattern. Administration was, however at the same slow rate during both control and experimental periods and there were no significant *changes* in PAH excretion during either type of respiratory stimulus when urinary acid base changes were maximal. PAH could also form a buffer pair but because it would be a strong acid buffer ($pK' = 3.83$) (13) such an effect must be very small.

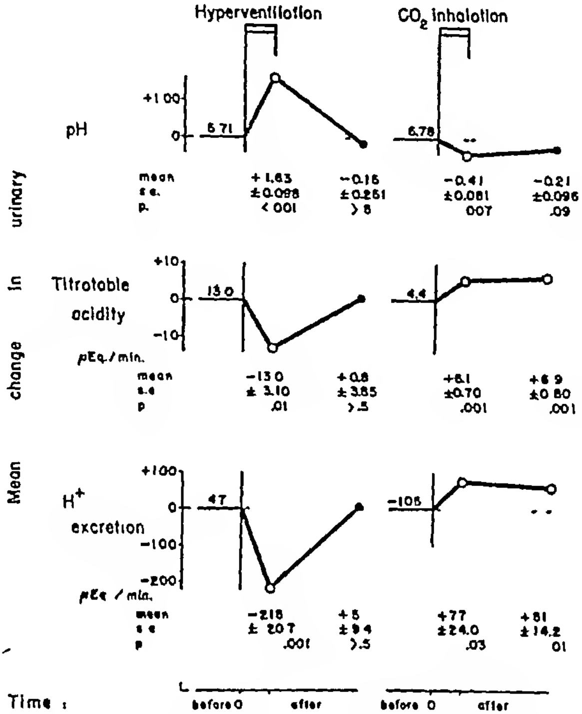

FIG. 1. ACUTE RESPIRATORY ALKALOSIS AND ACIDOSIS. MEAN CHANGES IN URINARY pH AND THE EXCRETION OF TITRATABLE ACID AND HYDROGEN ION

The mean for each group of changes from the individual mean control values is plotted for the end of the period of stimulus and the end of the experiment. The values for the mean change, its standard error and the probability of chance occurrence are given below the curves. The mean changes that are statistically significant ($p = 0.05$ or less) are represented by open circles. The abscissae represent time before, during and after the stimulus. The mean of the control values is given on the horizontal axis. Hydrogen ion excretion is defined as the sum of outputs of ammonium plus titratable acidity minus bicarbonate (see Equation 2 in text).

tion. The rate of renal adjustment to the disturbance by retaining H ion as compared to control H output (ΔUV_H by Equation 2) averaged 218 µEq per minute for the final period during hyperventilation. During the 30 minutes of the stimulus an average of 5.9 mEq H had been retained in this way. During hyperventilation the pH of the urine rose (mean = + 1.63 pH units) and titratable acidity fell (− 13.0 µEq per min.) both returning to the control values during the recovery period after hyperventilation. Bicarbonate excretion was markedly increased (+ 183 µEq per min.) and ammonium ion excretion decreased (− 30 µEq per min.) each subsequently re

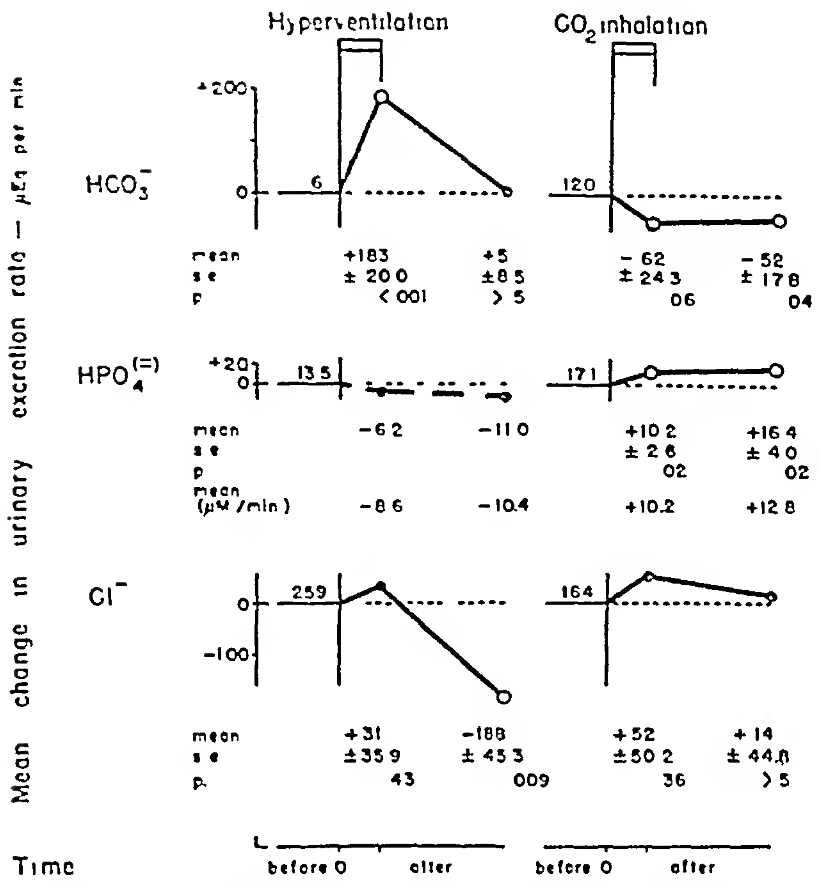

FIG. 2. ACUTE RESPIRATORY ALKALOSIS AND ACIDOSIS. MEAN CHANGES IN THE URINARY EXCRETION RATES OF ANIONS

The data are presented as in Figure 1. Since phosphate was measured in only two of the hyperventilation experiments, it is shown with a dotted line and statistical analysis omitted. The symbol $HPO_4^{(=)}$ indicates phosphate both as $HPO_4^=$ and $H_2PO_4^-$.

...ed to control rates. Potassium excretion in...sed markedly (+ 183 μEq per min), while ...her sodium nor chloride excretion showed a ...tically significant change. In the recovery ...od excretion rates fell significantly below con... for each of these ions (mean changes in rate ...r... K⁺ − 71 μEq, Na⁺ − 155 μEq, Cl⁻ ...88 μEq).

...ratory acidosis

...t is clearly shown by comparison of the magni...e of the estimated acid-base disturbance ...HCO_3^-... = 26 mEq) or of plasma acid-base ...(1), that CO_2 inhalation was a milder acid...d... hyperventilation. It is difficult to achieve a more severe acute experimental respiratory acidosis without undesirable side effects. Changes in urinary findings were correspondingly smaller during CO_2 inhalation than those observed during hyperventilation. ΔUV_{H^+} averaged 77 μEq per minute and cumulative H^+ eliminated during the stimulus was 1 9 mEq. Urinary pH fell (− 0 41 pH units) and titratable acidity increased (+ 6 1 μEq per min). Bicarbonate excretion decreased (− 62 μEq per min) in spite of a considerable increase in filtered load that resulted from the increased plasma concentration during CO_2 inhalation. The change in bicarbonate reabsorption (+ 310 μEq per min) is accordingly significant (p = 0 03). Thus a

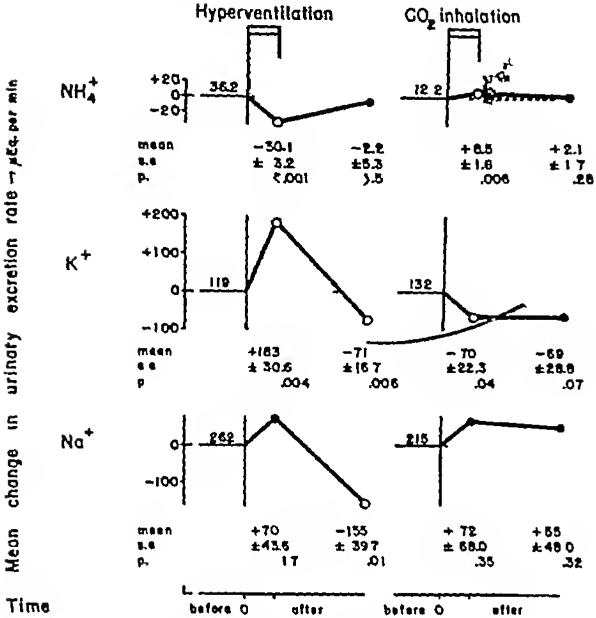

FIG. 3. ACUTE RESPIRATORY ALKALOSIS AND ACIDOSIS MEAN CHANGES IN THE URINARY EXCRETION RATES OF CATIONS

The data are presented as in Figure 1.

real physiologic response in bicarbonate regulation is evident, although if changes in excretion alone are considered the results just fail ($p = 0.06$) to show "statistical significance." Excretion of ammonium ion increased (+ 8.5 µEq per min.) and that of potassium decreased (− 70 µEq per min.) Neither sodium nor chloride output showed statistically significant change. Phosphate excretion was increased (+ 10 µEq per min.)

The small oral dose of sodium bicarbonate given preceding the respiratory acidosis experiments established a "baseline (a neutral or alkaline urine) upon which the effect of the CO_2 inhalation could be more readily determined Effects of the bicarbonate dose are evident in differences between the average control values in the respiratory alkalosis and acidosis experiments as shown in Figures 1 to 4 Four additional experiments

	PLASMA pH	URINARY HCO_3 EXCRETION	TUBULAR HCO_3 REABSORPTION	PLASMA pCO_2
RESPIRATORY ACIDOSIS	↓	↓	↑	↑
RESPIRATORY ALKALOSIS	↑	↑	↓	↓
METABOLIC ($NaHCO_3$ ADMIN.) ALKALOSIS	↑	↑	↑	↑

FIG. 4 CHANGES IN $NaHCO_3$ EXCRETION REABSORPTION AND RELATED EXTRACELLULAR FLUID (PLASMA) FACTORS IN ACUTE RESPIRATORY ALKALOSIS AND ACIDOSIS AND IN METABOLIC ALKALOSIS

Direction of change in response to the stimulus is indicated by the arrows. For each type of disturbance the plasma HCO_3 concentration changed in the same direction as the PCO_2. The rate of filtration of HCO_3 (or HCO_3 load) also changed in this direction.

TABLE I A

Respiratory alkalosis: Effects of hyperventilation on urinary pH, titratable acidity, and excretion of electrolytes

Expt and duration stimulus in min	Time*	pH	Titr acid	HCO3	Cl	NH4	Na+	K
	min		µEq per min	µEq per min	µEq per min	µEq per min	µEq per min	µEq per min
1	− 18							
	0	6.3		17	215	26	217	139
20	+ 29	7.8		251	212	7	219	114
	+ 61	7.6		101	79	8	65	189
	+ 89	7.2		123	64	13	90	151
	+119	7.2		64	56	10	89	97
3	− 53							
	− 31	5.70	10	9	195	42	230	116
	− 20	5.80	6	11	165	38	210	127
	0	5.88	5	11	172	35	225	123
30	+ 11	7.00	0	111	325	19	520	232
	+ 30	7.38	0	181	210	4	120	272
	+ 15	7.01	0	15	81	2	117	143
	+ 60	5.7	12	2	76	31	163	126
	+ 90	5.5	10	2	37	34	139	37
	+121	5.2	17	0	34	50	113	22
4	− 71							
	− 53	5.30	22	1	135	18	127	81
	− 37	5.39	18	2	116	54	104	92
	0	5.40	18	1	80	18	93	60
37	+ 22	6.77	4	64	170	24	160	167
	+ 37	7.20	0	171	251	13	214	332
	+ 55	—	1	16	72	5	55	130
	+ 86	5.31	10	0	59	27	31	60
	+117	5.2	15	0	34	31	32	47

Expt and duration stimulus in min	Time*	pH	Titr acid	HCO3−	Cl−	Phosphate		NH4+	Na	K+
	min		µEq per min	µEq per min	µEq per min	µEq per min	µM per min	µEq per min	µEq per min	µEq per min
5	− 17									
	− 33	5.57	23	4	187	23	22	36	252	105
	− 17	5.62	20	8	193	22	21	35	252	99
	0	5.62	22	7	212	21	20	35	263	99
32	+ 16	6.61	5	79	335	28	20	20	155	178
	+ 32	7.28	0	166	305	11	8	5	110	208
	+ 53	7.21	0	80	101	2.1	1.2	5	113	189
	+ 83	6.16	7	14	110	2.4	2.0	22	113	108
	+117	5.60	11	5	70	3.1	2.9	39	116	65
6	− 49									
	− 34	5.32	9	1	565			39	520	118
	− 19	5.10	10	2	640			17	580	168
	0	5.62	6	3	510			15	155	114
27	+ 16	7.04	0	132	610			19	600	292
	+ 30	7.35	0	218	185			6	500	370
	+ 51	6.68	0	36	325			14	258	186
	+ 82	5.55	5	2	285			34	288	57
	+116	5.22	9	0	176			11	178	38
7	− 55									
	− 38	6.24	4	3	258	4.6	4.7	18	278	109
	− 23	5.78	7	2	255	4.7	4.3	22	253	—
	0	5.28	15	1	250	5.0	1.8	31	220	118
58	+ 15	6.38	4	29	267	5.5	4.3	22	274	163
	+ 27	6.91	0	121	289	2.5	1.6	4	330	228
	+ 40	7.01	0	128	280	1.3	0.8	2	280	233
	+ 58	7.06	0	117	218	0.5	0.3	2	202	220
	+ 77	6.84	0	36	138	0.4	0.3	4	98	135
	+108	5.12	11	0	86	0.5	0.3	24	92	52
	+144	4.86	17	0	56	1.9	1.9	33	83	24

* Data are expressed per individual periods which end at the time indicated measured from the start of hyperventilation
Values obtained during hyperventilation are separated by horizontal single lines

TABLE I B

Respiratory acidosis Effects of CO_2 inhalation on urinary pH titratable acidity and excretion of electrolytes

Expt. and duration stimulus in min.	Time*	pH	Titr acid.	HCO_3^-	Cl^-	Phosphate	Phosphate	NH_4^+	Na	K
	min.		µEq per min	µEq per min.	µEq per min	µEq per min	µM per min	µEq per min.	µEq per min.	µEq per min.
1	− 37									
	− 25	7.00	7	56	296	29	18	23	352	83
	− 13	7.06	—	58	222	27	16	—	275	69
	+ 4	6.91	6	70	227	34	22	26	300	83
30	+ 14	6.50	6	50	243	36	27	39	373	49
	+ 33	6.34	14	60	469	48	39	36	634	39
	+ 48	6.52	9	74	400	47	35	22	592	42
	+ 68	6.52	12	25	292	38	28	26	400	27
	+ 97	6.18	21	9	217	36	30	42	311	16
2	− 57		†							
	− 41	6.93	4	245	164	23	15	10	275	199
	− 17	6.86	4	221	142	23	15	10	258	177
	− 2	6.83	—	181	133	—	—	9	235	156
27	+ 14	6.74	6	152	138	24	16	13	237	113
	+ 30	6.49	9	84	123	27	20	14	190	74
	+ 45	6.69	8	106	151	34	24	16	240	84
	+ 67	6.57	9	95	112	30	22	17	222	73
	+ 90	6.60	11	125	120	37	27	16	264	125
	+121	6.72	10	130	124	40	28	13	239	107
3	− 47		†							
	− 32	6.61	4	59	100	14	10	14	167	48
	− 16	6.82	2	84	104	9	6	11	182	64
	− 1	6.76	2	109	135	10	6	10	233	82
29	+ 13	6.97	2	104	152	14	9	9	252	85
	+ 29	6.59	7	70	214	22	16	17	282	58
	+ 44	6.64	7	67	209	24	17	14	280	64
	+ 63	6.73	8	111	317	31	21	12	425	90
	+ 97	6.73	8	124	352	33	23	12	480	100
	+129	6.73	9	97	305	37	25	13	415	92

Expt. and duration stimulus in min.	Time*	pH	Titr acid.	HCO_3^-	Cl^-	Phosphate	Phosphate	NH_4^+	Na^+	K^+
	min.		µEq. per min.	µEq per min.	µEq per min.	µEq per min	µM per min.	µEq per min.	µEq per min.	µEq per min.
4	− 47									
	− 32	6.72	4	116	137	4	3	9	186	129
	− 17	6.78	4	137	143	5	4	7	197	167
	− 2	6.73	6	119	169	6	4	7	216	155
21	+ 22	6.29	12	44	170	10	8	17	191	83
	+ 37	6.40	9	53	161	10	8	16	238	55
	+ 53	6.45	11	56	153	9	7	9	245	47
	+ 82	6.46	6	56	112	10	7	13	211	32
	+111	6.29	10	41	101	18	15	15	173	23
5	− 50									
	− 36	6.68	3	139	182	18	12	8	211	221
	− 20	6.58	2	111	158	15	11	7	181	189
	0	6.52	7	96	143	17	12	9	173	164
30	+ 15	6.46	6	73	124	22	17	9	157	116
	+ 30	6.17	11	29	100	29	24	20	138	58
	+ 44	6.27	20	36	150	43	35	20	193	68
	+ 61	6.45	18	53	144	46	36	13	206	86
	+ 92	6.51	16	67	160	51	38	12	250	82
	+122	6.57	14	62	135	39	28	8	223	62
6‡	− 51									
	− 35	5.88	12	10	56	11	10	40	33	105
	− 19	5.88	11	11	48	12	11	32	50	107
	0	5.94	13	14	58	14	13	32	56	118
22	+ 24	5.97	14	21	139	23	21	37	165	95
	+ 42	5.89	22	14	144	27	24	36	177	99
	+ 60	6.01	11	17	155	30	26	32	192	105
	+ 96	5.88	22	13	151	30	27	40	151	117
	+129	5.79	23	8	86	28	26	44	78	111

* Data are expressed per individual periods which end at the time indicated measured from the start of CO_2 inhalation. Values obtained during CO_2 inhalation are separated by horizontal single lines.

† Titratable acidity calculated from phosphate in Experiments 2 and 3

‡ Experiment 6 is not included in statistical analyses. This experiment was done as a control the urine being initially acid as explained in the text.

TABLE II

Respiratory alkalosis and acidosis. Calculated extracellular acid-base disturbance ($\Delta HCO_3^-{}_{e}$) and renal compensation (ΔUV_{H^+})† at end of respiratory stimulus*

A. Respiratory alkalosis (hyperventilation)				B. Respiratory acidosis (CO_2 inhalation)			
		ΔUV_{H^+}				ΔUV_{H^+}	
Exp.	$\Delta HCO_3^-{}_{e}$	Rate	Cumulative	Exp.	$\Delta HCO_3^-{}_{e}$	Rate	Cumulative
	mEq.	µEq. per min.	mEq.		mEq.	µEq. per min.	mEq.
3	−14.1	−21.5	−5.0	1	+4.6	+ 20	+0.64
4	−18.2	−22.6	−5.6	2	+1.2	+142	+4.3
5	−12.5	−21.2	−5.0	3	+3.8	+ 23	+0.16
6	−15.2	−29.2	−6.7	4	+ 2	+ 97	+2.3
7	− 6.7	−14.6	−7.0	5	+3.1	+105	+2.2
Mean	−13.5	−21.8	−5.9	Mean	+2.6	+ 77	+1.9

* $\Delta HCO_3^-{}_{e}$ indicates the total change in bicarbonate of the extracellular fluid (plasma plus interstitial fluid) and of the red cells (1).

† ΔUV_{H^+} indicates the change from control in urinary hydrogen ion excretion defined as the sum of ammonium plus titratable acid minus bicarbonate output (see Equation 2 in text).

were done without any disturbance of respiration to evaluate the effect of the oral bicarbonate alone. These experiments also provided a control for the acidosis series with respect to the effects of the diurnal "tide" and the water loading procedure. While slight apparent changes were present at the times that would correspond to the respiratory stimulus none of them was statistically significant. Changes during the stimulus in the respiratory acidosis series which are significantly different from the preceding control values also differ significantly from the slight alterations at corresponding times in the control series. This is true for all of the variables except urinary pH and K excretion, the differences of which were not quite significant at the 5 per cent level. During the comparatively longer recovery portion of the experiments, a tendency in the acidosis series to disappearance of the effects of the oral bicarbonate on the "baseline" would be indistinguishable from persistence of acidification of the urine as a compensatory mechanism to the respiratory disturbance. Accordingly discussion of changes late in recovery is limited to respiratory alkalosis. Results are not reported in detail for the control series.

[illegible] and urinary volume

Measurements of the effective renal plasma flow, glomerular filtration rate and rates of urine flow are not reported in detail. Significant changes were observed only with hyperventilation. Effective renal plasma flow was depressed from control at the end of these experiments, a change correlating with the excretion rate of sodium and chloride which were also significantly depressed at this time. Glomerular filtration rate was also decreased significantly at the end of the experiments, but also decreased somewhat during the hyperventilation, at a time when excretion of sodium and chloride and effective plasma flow were not significantly altered and if anything were a little increased. Changes in urine flow were probably more closely related to the water loading than to the experimental procedure.

DISCUSSION

Observation of renal acid-base adjustment

The kidney responds promptly to primary respiratory alterations of acid base equilibrium. The renal adjustment is not a simple correction of the abnormality present, but may be considered as eventually producing an opposing "metabolic" (as contrasted to "respiratory") disturbance which may be measured as a change in the rate of hydrogen ion excretion (ΔUV_{H^+}). An increase in UV_{H^+} is equivalent to an increase in buffer base in the body. Singer and Hastings (14) suggested certain practical advantages in focussing attention on the buffer base concentration as a quantitative index of the metabolic or non-respiratory factor in acid base equilibrium and pointed out that a pure respiratory disturbance (before various com-

TABLE III

Respiratory alkalosis and acidosis Renal filtration reabsorption and excretion of bicarbonate

				Plasma			HCO_3^-			
		Urine flow	GFR				Filt.	Excr	Reabsorbed	
Expt.	Time*	*ml per min.*		pH	PCO_2 *mm. Hg*	HCO_3^-† *mEq per L.*	*mEq per min.*	*mEq per min.*	*mEq per min.*	*mEq per L. filtrate*
				A. Respiratory alkalosis—hyperventilation						
3	C*	10.4	125	7.38	43	24.9	3.11	.010	3.10	24.82
	S	5.0	109	7.62	19	19.2	2.09	.184	1.91	17.52
	R	1.9	124	7.40	42	25.4	3.15	0	3.15	25.42
4	C	6.6	104	7.35	43	23.6	2.45	.001	2.45	23.58
	S	6.2	108	7.65	15	16.5	1.78	.171	1.61	14.92
	R	0.5	80	7.43	37	24.4	1.95	0	1.95	24.41
5	C	12.1	122	7.39	43	25.6	3.13	.006	3.12	25.58
	S	5.3	87	7.63	19	20.2	1.76	.166	1.59	18.29
	R	9.3	118	7.44	39	25.7	3.03	.005	3.03	25.67
6	C	7.5	109	7.39	43	25.3	2.76	.002	2.76	25.28
	S	7.8	86	7.66	16	17.9	1.54	.248	1.29	15.00
	R	3.8	96	7.43	37	24.2	2.32	0	2.32	24.20
7	C	1.6	132	7.30	44	24.9	3.29	.002	3.29	24.89
	S	5.9	108	7.56	25	22.2	2.40	.117	2.28	21.13
	R	3.9	109	7.39	42	24.9	2.71	0	2.71	24.89
				B. Respiratory acidosis—CO_2 inhalation						
1	C	3.0	139	7.40	46	27.5	3.82	.061	3.76	27.05
	S	17.2	151	7.35	54	29.9	4.52	.060	4.46	29.53
	R	4.0	135	7.45	40	27.0	3.64	.010	3.64	26.97
2	C	18.2	126	7.46	40	27.8	3.50	.216	3.29	26.11
	S	16.4	127	7.40	47	28.7	3.64	.123	3.52	27.71
	R	17.2	131	7.43	40	26.5	3.47	.125	3.35	25.57
3	C	11.1	111	7.50	34	25.5	2.83	.084	2.75	24.76
	S	10.6	114	7.37	48	27.1	3.09	.070	3.02	26.49
	R	13.6	117	7.44	37	24.4	2.86	.111	2.74	23.42
4	C	16.7	115	7.44	41	26.7	3.07	.124	2.95	25.63
	S	14.8	117	7.34	51	27.0	3.16	.044	3.12	26.68
	R	15.8	116	7.48	34	24.6	2.85	.056	2.80	24.13
5	C	16.8	108	7.44	38	25.3	2.73	.115	2.62	24.26
	S	13.1	107	7.33	52	26.5	2.84	.029	2.81	26.18
	R	14.1	106	7.44	26	24.2	2.57	.053	2.51	23.68
6‡	C	12.1	102	7.32	49	24.7	2.52	.012	2.51	24.60
	S	15.3	110	7.26	62	27.1	2.98	.021	2.96	26.90
	R	14.7	106	7.37	45	25.5	2.70	.013	2.69	25.38

* Time of individual periods and respiratory stimuli is given in Table I A and I B
C denotes average of the control periods.
S denotes the last period during the respiratory stimulus
R denotes the last period for which complete data were available during recovery from the effects of the stimulus

† The plasma concentration of HCO_3^- given and used to calculate HCO_3^- filtered is that observed (often at the end of the period) rather than an interpolated value for mid period No correction is made for serum water or Donnan factor

‡ Experiment 6 during CO_2 inhalation was done as a control the urine being initially acid as explained in the text.

pensations) causes no change in whole blood buffer base. Fuller and MacLeod (15) have calculated the rate of "total H⁺ secretion" by adding HCO_3^- *reabsorption* to titratable acidity plus ammonium excretion rather than by subtracting HCO_3^- *excretion* as we do in calculating UV_{H^+}. The resulting index is quite different since our index indicates the net *effective acid base adjust*

ment accomplished by the over-all process of urine formation while theirs relates to the specific process of H^+ secretion by the tubular cells.[7]

In *respiratory alkalosis* the characteristic response is a decrease in H^+ output measured principally by an increase of HCO_3^- with fixed cation in an alkaline urine (17–22). Associated with this is a decrease in output of ammonia and titratable acid. The resultant effect on total body fluids is a loss of buffer base, a further reduction of the already lowered HCO_3^- concentration and a return of pH toward the normal range. The accompanying predominant change in excretion of "fixed" ion in the first half hour of acute experiments is increased K^+ loss, rather than change in Na^+ or Cl^-. The duration of the experiments was such that cumulative excretion while the stimulus persisted had very little effect in changing extracellular or total body fluid buffer base. The renal compensation was therefore small compared to the rapid sharing of the effects of the stimulus by the various "buffer" mechanisms of the body previously estimated in detail (1). Calculations based on the data of other workers (23, 24) indicate that in more prolonged respiratory disturbances the major part of $\Delta HCO_{3\,ecf}^-$ may be compensated by cumulative urinary changes.

Renal compensation for acute respiratory alkalosis appears to be less efficient than that for acute metabolic alkalosis of the "electrolyte addition" type. In seven experiments in which we gave rapid intravenous infusions of hypertonic sodium bicarbonate (25, 26) the total dose of bicarbonate given was of the same order of magnitude as the $\Delta HCO_{3\,ecf}^-$ observed in respiratory alkalosis reported here. Yet, within the same time cumulative ΔUV_H averaged 19.6 mEq, indicating more than three times the comparable renal compensation during respiratory alkalosis.[8] The difference may be related to the fact that this type of metabolic alkalosis (a simple excess of sodium and bicarbonate) may be corrected by the renal excretion of the ions present in excess.

The characteristic changes in *respiratory acidosis* are the opposite of those described for respiratory alkalosis (15, 17, 27). There is an increase in excretion rates of H^+, titratable acid and ammonia and a decrease in excretion of bicarbonate with fixed cation, especially potassium. The urine becomes more acid. Since the acid-base disturbance of acute experimental CO_2 inhalation is considerably milder than that of hyperventilation, the urinary changes are much smaller in absolute terms. Relative to the estimated acid-base disturbance ($\Delta HCO_{3\,ecf}^-$), however, the renal response appears to be of the same order of magnitude. When the experiments were planned we postulated that it would be difficult to detect an increase in UV_{H^+} if the control urine were already acid. If the "basal" state of the kidney involves compensation for the tendency of the usual diet to produce a slight metabolic acidosis, it would scarcely be surprising that an acute respiratory acidosis of mild degree should fail to bring about a further detectable increase in UV_{H^+}. We believe that this is the explanation for the lack of response in urinary acid-base factors noted by Longson and Mills (28), and in urinary output of sodium, potassium and chloride by other workers (29), during acute CO_2 inhalation in man. Similarly, in our Experiment 6, with NaCl substituted for the small pre-medication dose of $NaHCO_3$ (and a control urine pH of 5.9) there was no detectable change in UV_{H^+}. In spite of the acid control urines, it seems probable that a renal tubular response could have been found in the experiments of Longson and Mills (28) had changes in filtered load and reabsorption of bi-

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[7] The use of the index proposed by these authors involves the assumption that the basic tubular process of H^+ secretion is involved in the entire quantity of HCO_3^- reabsorbed. This view is presently held by most workers in this field but it has also been recently challenged (16).

[8] For the hyperventilation experiments renal adjustment was calculated by an alternative approximation rather than by ΔUV_H as defined in this paper. From the law of electroneutrality it follows that changes in UV_H must be accompanied by an equal change in excretion of fixed cation minus fixed anion of opposite algebraic sign. Under many circumstances, including our own experimental conditions, a fairly satisfactory approximation of ΔUV_H can be derived from changes in excretion rates of the major fixed ions of urine as $Na + K - Cl$. Expression of the urinary adjustment in these alternative terms directs attention to the effect on body fluid content of the major fixed ions which may be convenient in consideration of acid-base changes there. Use of this approximation during hyperventilation indicates an average change of $+222$ μEq per min, surprisingly close to the ΔUV_H of -218 μEq per min by the other method.

carbonate been determined rather than in excretion alone. Data of Denton Maxwell McDonald, Munro, and Williams (30) during CO_2 inhalation in sheep indicate a marked rise in HCO_3^- reabsorption, although like Longson and Mills, they observed no change in rate of urinary excretion of HCO_3^- In contrast to these negative urinary results other workers have found characteristic changes in urinary acid base factors in acute respiratory acidosis in dogs (15 17 31)[9] Where given control urinary pH values in these experiments were close to 7 0 or above. We observed significant urinary changes in the five subjects who were given the preceding oral dose of $NaHCO_3$ and in whom the control urine pH was also approximately neutral

Other factors influence the characteristic renal response observed during respiratory acid base disturbances For example, the typical urinary response to respiratory alkalosis is lacking in subjects who are in a state of NaCl depletion (18 20) It is to be expected that pre-existing disturbances of the circulation or fluid and electrolyte balance endocrine factors and kidney disease may alter the characteristic changes that have been defined

In more prolonged respiratory disturbances (for example of four or five days duration) the ability of the kidney to compensate appears increased over that seen initially (32) Ammonium excretion is likely to constitute a larger fraction of the renal response to more prolonged acidosis Although NH_4 output begins to rise within a few minutes of onset of acidosis it does not approach maximal rates for some hours or days (33)

The urinary findings in different phases of respiratory acid base disturbances

It must be emphasized that the characteristic combination of urinary changes described above is typical only of the displacement' phase of the respiratory disturbance. While changes in urinary rates of H excretion persist they result in a progressive increase in the degree of metabolic acidosis or alkalosis which compensates in part for the respiratory disturbance. If the abnormal level of PCO_2 persists for periods much longer than the duration of the present experiments eventually a point will be reached where the secondary change in extracellular buffer base and other chemical changes have restored the pH to a level of stability in or closer to the normal range. In the ensuing steady state' phase of the disturbance renal compensation ceases in the sense that no further progressive change is produced in buffer base of body fluids Output of H then reflects the dietary intake and other physiological processes as it does in a state of normal respiration and PCO_2 Finally during the "recovery phase of a chronic respiratory acid base disturbance, when PCO_2 is returning toward normal, renal compensation serves to restore the extracellular buffer base to normal Changes are therefore the precise opposite of those described for the displacement phase increase in UV_H in chronic respiratory alkalosis and decrease in chronic respiratory acidosis Thus in chronic respiratory disturbances a temporary increase in the respiratory difficulty (equivalent to an additional displacement' phase) will be accompanied by reappearance of characteristic urinary changes a temporary decrease in the respiratory difficulty (equivalent to a partial recovery phase) will cause the opposite type of urinary changes Such a sequence of events is quite otherwise to the situation found in metabolic acid base disturbances In a complete study of the displacement, stabilization, and recovery phases of ammonium chloride acidosis Sartorius Roemmelt, and Pitts (33) have shown that the fundamental urinary response is one of increased UV_H in all of these phases despite serial differences in individual constituents that are also of importance. The same holds true for the opposite changes in metabolic alkalosis produced by electrolyte addition (25, 26) In acute respiratory experiments of brief duration such as those reported here there is no stabilization and recovery is marked simply by a return of the urinary acid base output toward control levels[10] Superimposed on this return

[9] A change is evident in the urinary data of Fuller and MacLeod (15) although their calculation of "total H secretion" did not change significantly because of a fall in glomerular filtration rate and therefore in bicarbonate reabsorption. Their method of calculation showed that 90 to 98 per cent of "total H secretion" was accounted for by bicarbonate reabsorption in respiratory disturbances.

[10] Fuller and MacLeod (15) reported that urinary effects of respiratory acidosis were rapidly reversible,

[illegible] are the effects of other factors such as d[illegible] (34), the wearing off of the [illegible] metabolic alkalosis previously induced in [illegible] subjects exposed to CO_2 inhalation persistence of slight differences in respiration and late effects of the imposed diuresis.

Once the "balance" between the existing respiratory disturbance and the compensatory metabolic acidosis or alkalosis (produced by the kidney) has been reached there similarly may be nothing distinctive about the urinary excretion for individual ions except as related to the accompanying composition of plasma. For example, in one of our unpublished cases of chronic CO_2 retention with a plasma PCO_2 of 120 mm. Hg, urinary chloride excretion exceeded 40 mEq. per day, corresponding approximately to the intake, despite an extremely low plasma chloride concentration of 74 mEq. per liter. It is not surprising that the observer may erroneously conclude that renal compensations are unimportant in such disturbances if he directs his attention to the urinary composition alone. Determination of clearance rates or reabsorptive rates for acid-base factors will, however, disclose the presence of renal response.

Regulation of renal bicarbonate excretion

Change in the rate of excretion of bicarbonate was the principal anionic response of the kidney to the respiratory disturbances. In a preliminary report of the present work (3) it was stated that [illegible] rate *excretion* rate correlated better with plasma pH than with other plasma factors such as PCO_2 or HCO_3^- concentration. About the same time Brazeau and Gilman (35), Dorman, Sullivan and Pitts (26) and Relman, Etsten and [illegible] (37) each reported studies showing that the *reabsorption* of bicarbonate was more closely [illegible] to the PCO_2 of the blood than to pH or [illegible] bicarbonate. Several recent references [illegible] our data and interpretations [illegible] with the above groups. This [illegible] resulted from the failure to note the terms in which the data are reported. Examination of the directional changes given in Figure 4 will disclose that actually no disagreement exists. In our consideration of the over-all effect of the renal compensation on the body we stressed output (excretion). The figure illustrates that HCO_3^- excretion is decreased in respiratory acidosis and increased in both respiratory and metabolic alkalosis. Since both plasma PCO_2 and plasma HCO_3^- concentration move in the opposite directions in respiratory alkalosis and metabolic alkalosis, it is obvious that HCO_3^- *excretion* is better correlated with plasma pH. The three groups of investigators mentioned above, however, were interested primarily in the renal component due to active tubular processes. In studying the regulation of these processes they therefore logically stressed bicarbonate *reabsorption*. Figure 4 shows that the rate of HCO_3^- reabsorption is decreased in respiratory alkalosis but increased in metabolic alkalosis (electrolyte addition). Obviously, then, reabsorption does not correlate well with plasma pH but could be correlated (in direction of change) with either plasma bicarbonate concentration or PCO_2.[3] In experiments carefully designed to test this point the groups mentioned above found in dogs that PCO_2 was the more closely related. If our human data are used for calculation of correlation coefficients it is found that reabsorptive rate does correlate somewhat better with PCO_2 than with plasma bicarbonate concentration. Thus, it should be apparent that conclusions about changes in bicarbonate *excretion* cannot be considered as simply the opposite of changes in *reabsorption*. If the filtered load changes sufficiently, rates of reabsorption and excretion may change simultaneously in the same direction as happened in the bicarbonate administration experiments.

Renal excretion of potassium

The predominance of changes in potassium excretion rate over those of sodium is a striking result since the sodium greatly exceeds potassium in the glomerular filtrate. At the end of either hyperventilation or CO_2 inhalation the significance of change in potassium excretion compared to con

[illegible] depression of [illegible] bicarbonate ex[illegible] experiments [illegible] after hyperven[illegible] and those of San[illegible] 2

[3] Several workers have advanced the suggestion that this effect may be mediated through changes in pH within the tubule cells (38, 39).

trol rate clearly exceeded that of the corresponding changes in sodium excretion Indeed in the hyperventilation experiments even the absolute magnitude of increased excretion was much greater (K + 183 μEq per min. Na^+ + 70 μEq per min.) Since potassium is the predominant intracellular cation one might suggest that the release of potassium from body cells as a compensatory mechanism to hyperventilation is involved A close relation between potassium and buffering action of intracellular fluid has been prominently considered since the work of Darrow and his associates (40, 41) on certain potassium deficiency states The calculated 'cellular exchanges of potassium in our experiments (1) would affect extracellular concentration in the same direction as do the changes in urinary excretion of potassium Thus they are in the direction opposite to that which would be required to explain the urinary findings through a change in plasma potassium concentration and secondarily in renal load. The change in potassium excretion under these circumstances might result from a reciprocal relationship between the tubular secretions of hydrogen ion and potassium as suggested by Berliner, Kennedy, and Orloff (42)

Sodium and chloride excretion

In extracellular fluid changes in buffer base are approximately equal to changes in Na $-$ Cl^- Yet during the first half hour of respiratory acid-base disturbances those changes that did occur in sodium and chloride excretion tended to be in the same direction. Therefore urinary Na $-$ Cl^- excretion showed little variation and change in fixed ions accompanying ΔUV_{H^+} was associated predominantly with K The alterations in sodium and chloride excretion were not directly correlated with the continuation and withdrawal of the experimental stimuli and were not necessarily opposite in direction in the acidosis from the alkalosis experiments. Our data therefore do not support the suggestion of Stanbury and Thomson (20) that a fall in chloride output following acute hyperventilation may represent a separate acid base mechanism favoring conservation of fixed acid" (Cl^-) Since these parallel changes of sodium and chloride excretion are not concerned with preserving acid base homeostasis some other physiological process must be involved. This might concern mechanisms of electrolyte conservation, hormonal factors, or renal hemodynamic changes. Renal plasma flow, but not glomerular filtration rate, showed a positive correlation with sodium and chloride excretion in the hyperventilation studies while neither showed statistically significant changes with CO_2 inhalation.

Excretion of other ions

Phosphate excretion changes in two ways as the rate of total phosphate output (in μM per min) and as the proportion as $HPO_4^=$ or as $H_2PO_4^-$ Changes in the buffer role of phosphate as a transporter of H are included in the titratable acidity of which phosphate is an important part. This may be expressed quantitatively by the difference between the excretion rate of phosphate in μEq per min. at the observed pH and the calculated rate in μEq per min that would occur if the same number of μM were excreted at a pH of 7 4 An increase in μM per min phosphate excretion is regularly observed in respiratory acidosis (17 30, Table I B) and a decrease in respiratory alkalosis (17, 19 20, Table I A) but these changes are small relative to the other changes Calculated *undetermined anion* excretion showed only very small changes in both types of experiments It is therefore unlikely that changes in *excretion* of sulfate, lactate or organic acids are quantitatively important compared to the other changes reported

SUMMARY

Acute respiratory alkalosis by voluntary hyperventilation for approximately thirty minutes or acidosis by CO_2 inhalation for a similar period were induced in normal human subjects The urinary excretion of water and electrolytes and the acid base pattern of the urine were observed in multiple clearance periods before, during and after the respiratory stimuli

In *respiratory alkalosis* the kidney responded promptly by retaining hydrogen ion compared to control excretion, measured principally as an increase in output of bicarbonate with potassium Urinary pH rose and titratable acidity ammonium ion, and phosphate excretion fell The potassium effect appeared to be due to renal regulation rather than secondary to systemic intracellular adjust

ments Chloride excretion tended to vary with that of sodium, increasing slightly during hyperventilation and then falling far below the control level

Changes observed during *respiratory acidosis* were, for most variables, opposite in direction to those noted during hyperventilation They were smaller in magnitude since the experimental acute respiratory acidosis by CO_2 inhalation was a milder acid-base disturbance than hyperventilation as indicated by degree of plasma changes and estimation of total extracellular acid-base disturbance Changes in excretion of sodium and chloride were not as definite as those in other factors and were not always opposite in direction in the two types of experiment. At least part of this change (when sodium and chloride change in the *same* direction) appears not immediately concerned with acid-base homeostasis If the urine is already acid the typical urinary changes may not be evident during acute CO_2 inhalation

Renal mechanisms account for only a small part of the adjustments observed in experiments of short duration, but become more important with prolonged stimuli The rate of renal acid-base compensation is considerably greater in acute extracellular alkalosis of similar magnitude induced by sodium bicarbonate administration The urinary patterns described are typical of the "displacement" phase of respiratory disturbances With chronic respiratory disturbances a stabilized situation may be reached in which changes are not evident in rates of urinary output of hydrogen ion except as compared to abnormalities of the acid-base composition of plasma, if "recovery" then occurs the direction of hydrogen ion excretion typical of the "displacement" phase is reversed

The data are compatible with the finding of others that the tubular *reabsorption* of HCO_3^- is better correlated with plasma PCO_2 than with other extracellular acid-base factors Changes in *excretion* of HCO_3^-, however, which determine the effect on the body, correlate with plasma pH and not with plasma PCO_2, if one considers both respiratory and metabolic disturbances

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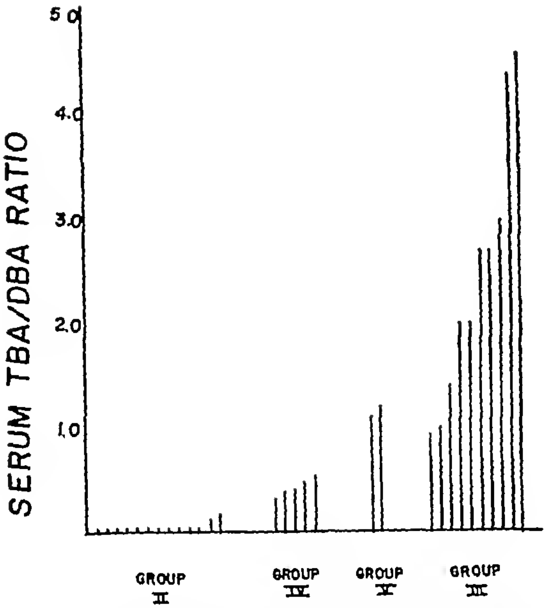

FIG 1 SERUM TBA/DBA RATIO AMONG THE VARIOUS GROUPS OF PATIENTS WITH HEPATIC DISEASE

and serum cholesterol within normal limits,[3] and negative history for alcoholism, dietary imbalance, or hepatotoxic agents Liver biopsies substantiating the diagnosis of chronic hepatitis were performed on Cases Nos 34 and 35 (Table I), biopsy was not done on the other three patients in this group

Group V Two cases of acute hepatitis One patient was a 25-year-old woman with acute hepatitis, presumably of viral etiology, who made an uncomplicated recovery Liver biopsy was not performed in this patient. The second subject was a 64-year-old man who became jaundiced two months after commencement of the administration of a testosterone analogue. Liver biopsy indicated acute liver cell injury Following discontinuance of the drug, the findings of liver damage rapidly disappeared.

METHODS

Analysis was carried out on a 4 to 15-ml sample of fresh serum Bile acid concentration was found to be unaffected by preservation of serum for several months at −20° C. An alcoholic extract of the serum sample was made as described by Josephson (3) The extract, after removal of the barium salts and neutralization, was concentrated to a volume of about 30 ml on the steam bath. An equal volume of H_2O was added, the pH was adjusted to 9, and the solution was extracted three times with petroleum ether (B P 68° C) to remove triglycerides and cholesterol The aqueous solution was neutralized and evaporated to dryness The bile acid content of this residue was determined by one of two procedures

In one procedure, used for the determination of the total bile acid content, the residue was dissolved in 20 ml. 5 per cent NaOH and hydrolyzed by heating for 3 hours in an autoclave at 15 lbs pressure. After cooling, the aqueous solution was made acid to Congo Red with concentrated HCl and extracted five times with equal volumes of ethyl ether After being dried over Na_2SO_4 the ethereal solution was taken to dryness The residual material was fractionated by partition chromatography according to the method of Mosbach, Zomzely, and Kendall (7) The first fraction (100 per cent petroleum ether) was discarded. The second and third fractions (60 per cent petroleum ether, 40 per cent isopropyl ether, and 40 per cent petroleum ether, 60 per cent isopropyl ether, respectively) were taken to dryness and acetic acid was removed by repeated evaporation after the addition of benzene. Aliquots from fraction 2 were analyzed spectrophotometrically for total dihydroxy bile acid and for chenodeoxycholic acid (7, 12), and from fraction 3 for trihydroxy bile acid.

In the other procedure, used when information concerning the conjugation of the bile acids was desired, the residue was taken up in 50 ml of ethanol and the insoluble portion removed by filtering Aliquots of the ethanolic solution, after acidification, were fractionated by the reverse phase chromatographic systems of Norman (13) Appropriate fractions from the column were hydrolyzed, acidified to Congo Red, and free bile acid recovered by ether extraction. Di- and tri hydroxy bile acids were then determined spectrophotometrically

The bile acid content of a 50 ml aliquot of urine was determined as follows After the addition of 2 5 gm. of NaOH, hydrolysis was carried out in the autoclave at 15 lbs for 3 hours The solution was treated thereafter exactly as described for the hydrolysate of the material derived from serum

RESULTS

Serum and urine bile acid content

Table I gives data on the bile acid content of the serum and urine of the 40 subjects, together with other laboratory findings pertinent to the hepatic disease Figure 1 illustrates the distribution of the serum trihydroxy/dihydroxy bile acid ratios among the four groups of patients with hepatic disease

No bile acid could be detected in the serum or urine of any of the subjects without hepatic disease (Group I)

In 12 of 13 patients with Laennec's cirrhosis (Group II), the serum contained dihydroxy bile acid (DBA) in amounts of 0 6 to 5 0 mg per cent Trihydroxy bile acid (TBA) was detectable in only 2 of these cases, at a level of 0 4 and 0 6 mg

[3] Case No 38 (Table I) in this group exhibited, during certain periods of his illness, a borderline elevation of the serum cholesterol and alkaline phosphatase.

TABLE II

Serial bile acid determinations

Case No	Date	Serum DBA mg. %	Serum TBA mg %	Bilirubin mg %	Comment
13	7/28/55	6 2	0	5 5	Laennec s cirrhosis status quo
	8/8/55	7.2	0	5.5	
	9/19/55	5 0	0 6	4 5	
	9/28/55	5 4	0	4 7	
	10/11/55	5 9	0	4 7	
15	1/9/56	2 5	0 4	4.3	Laennec s cirrhosis gradually receding jaundice
	2/15/56	1 7	0	2 4	
	3/12/56	1 7	0	2.1	
38	5/1/56	3 0	2 7	10.2	Chronic hepatitis in exacerbation death on 5/26/56
	5/4/56	3 5	1 4	10 9	
	5/10/56	5 0	2 9	11 6	
40	8/26/55	4 1	4 4	24 8	Acute viral hepatitis complete recovery
	9/7/55	2 5	3 6	11 0	
	9/21/55	1.3	0	3.2	
	9/30/55	0	0	0.8	

per cent. The one subject (Case No 21) in whom dihydroxy bile acid was not detectable in the serum was in the terminal stage of hepatic coma at the time of the analysis following hemorrhage from esophageal varices Urinary bile acid was determined in 8 individuals in this group In 2 cases dihydroxy bile acid was present in the amounts of 4 0 mg , and 7.3 mg per 24 hrs

In all 10 cases of obstructive jaundice (Group III), the serum contained both di and trihydroxy bile acid TBA concentration exceeded that of DBA in 8 cases The ratio of TBA to DBA serum concentration varied from 0 9 to 4 6 Five of the 6 patients whose urine was analyzed excreted both types of bile acids in the urine The 24-hr bile acid excretion in the adult patients varied from 9 0 mg to 28.4 mg for DBA from 25 0 mg to 26 0 mg for TBA.

The serum of all patients with chronic hepatitis in Group IV contained both di and trihydroxy bile acid Dihydroxy bile acid concentration exceeded that of trihydroxy bile acid the TBA/DBA ratio varying from 0.3 to 0 5 Bile acids were detected in the urine in all cases studied

In one case of acute viral hepatitis (Group V) on the 20th day of the illness the serum DBA was 4 1 mg per cent and the TBA 4 4 mg per cent. In a case of acute hepatic injury due to drug toxicity serum DBA was 1.2 mg per cent and serum TBA 1 4 mg per cent.

Serial determinations of serum bile acids

In 4 patients the bile acid concentrations were determined repeatedly during the hospital course (Table II) There was little variation in the concentration of serum DBA in 2 patients with Laennec's cirrhosis who exhibited little change in clinical condition during the intervals encompassed by these determinations

On the other hand in a man with chronic hepatitis, the serum bile acids were determined three times during an exacerbation of his disease The serum DBA level rose steadily while the patient s condition worsened with progressive lethargy and the onset of neurological symptoms which culminated in coma and death

The serum of a 25-year-old woman with acute viral hepatitis was analyzed three times for bile acids during the favorable course of her illness The decrease in serum bile acids paralleled the rapid clinical improvement

Observations on the structure of the serum bile acids

Since the foregoing analyses of hydrolyzed extracts of serum provided no information concerning the state of conjugation or mode of transport of the serum bile acids and since these details are crucial in the consideration of the mechanisms which underly the presence of bile acids in the

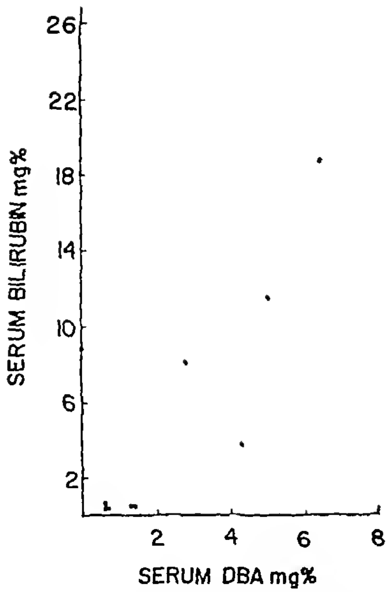

FIG 2 RELATIONSHIP OF SERUM DIHYDROXY BILE ACID CONCENTRATION TO SERUM BILIRUBIN CONCENTRATION IN PATIENTS WITH HEPATIC DISEASE

normal values for cephalin flocculation or albumin-globulin ratio The patients with appreciable serum levels of trihydroxy acids tended to have high values for serum alkaline phosphatase These patients also exhibited high serum cholesterol levels In all types of liver disease studied, high serum levels of bile acid tended to be associated with high values for serum bilirubin (Figure 2) In our series the correlation between these values was better for the patients with Laennec's cirrhosis, where practically all the serum bile acid was of the dihydroxy type, than for the patients with biliary obstruction

In the patients with Laennec's cirrhosis, the serum DBA level did not reflect the over-all severity of the disease Several patients with Laennec's cirrhosis advanced enough to produce neurological symptoms (Cases Nos 11, 17), severe portal hypertension (Cases Nos 16, 17, 20, 21) or marked hypoalbuminemia (Cases Nos 12, 21, 22), exhibited relatively low serum DBA Although serum DBA concentration showed little fluctuation in the individual cirrhotic patient (Table II), it was highly variable from one patient to another Serum DBA concentration undoubtedly is determined by the interplay of several factors, including the rate of conversion of cholesterol to bile acid and the concentration of transporting plasma proteins

Excretion of bile acids in the urine

Bile acids were found in the urine of all patients with obstructive jaundice having appreciable serum levels Bile acids were not found in the urine of most patients with Laennec's cirrhosis The presence of bile acid in the urine of two cirrhotic subjects (Cases Nos 18 and 19) may be related to a moderate proteinuria which was present in both of these patients

The mechanism of renal clearance of these substances appears to be glomerular filtration followed by extensive tubular reabsorption At a serum concentration of 5 mg per cent approximately 76 per cent of TBA and 95 per cent of DBA is bound to serum albumin Only the unbound serum bile acid would be filtered by the glomeruli On the basis of serum concentrations, binding constants (15) and urinary bile acid content, it was calculated that more than 95 per cent of the filtered bile acid was reabsorbed by the tubules in several patients with obstructive jaundice

Preliminary observations have indicated that the bile acids in both the urine and serum of patients with obstructive jaundice are largely in conjugated form In patients with Laennec's cirrhosis, only a small portion of the serum bile acid is conjugated Differences in the state of conjugation of the bile acids may affect their renal tubular reabsorption Further study of the nature of the bile acid conjugates in serum and urine will be necessary, in order to understand the differences in urinary excretion of bile acids in these patients

SUMMARY

In normal subjects, bile acids were not detectable in the serum or urine In 12 of 13 patients with Laennec's cirrhosis, 0 5 to 5 5 mg per cent of dihydroxy bile acid (DBA) was present in the serum In only two of these patients was trihydroxy bile acid (TBA) detectable (0 4 to 0 6 mg per cent) Only a small proportion of the serum dihydroxy bile acid from patients with Laennec's cirrhosis was conjugated Impaired activity of the conjugating and hydroxylating enzymes within

the liver cells probably accounts for the serum bile acid findings in this group

All of 10 patients with biliary obstruction were found to have an accumulation of both tri and dihydroxy bile acids in the serum The ratio of tri to dihydroxy acid varied from 1 to 4 In these patients the serum bile acids were largely conjugated with glycine and taurine, and probably accumulated in the blood as a result of regurgitation from the biliary passages.

The following correlations were observed between serum bile acids and the other findings of hepatic disease in these patients

1) The serum TBA/DBA ratio indicated the relative intensities of biliary regurgitation and of hepato-cellular injury in the hepatic disorder

2) Serum DBA concentration exhibited a correlation with serum bilirubin in both hepato-cellular and regurgitative jaundice

3) In patients with a component of biliary obstruction, the serum TBA concentration was proportional to the hypercholesterolemia.

Bile acids were regularly excreted in the urine by patients with obstructive jaundice but were not detected in the majority of patients with Laennec's cirrhosis Differences in the state of conjugation of the bile acids appear to affect the renal clearance of these substances

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BILE ACID CONTENT OF HUMAN SERUM II THE BINDING OF CHOLANIC ACIDS BY HUMAN PLASMA PROTEINS [1]

By DANIEL RUDMAN [2] AND FORREST E. KENDALL

(From the Columbia University Research Division, Goldwater Memorial Hospital and the Department of Medicine, Columbia University, New York, N Y)

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Although many observations indicate that bile acids and their derivatives may be bound by serum proteins, little information upon the quantitative aspects of this reaction is available Lecomte du Noüy (1) observed that the activity of bile salts in lowering the surface tension of aqueous solutions was suppressed by the presence of serum

The activity of human albumin in preventing the lysis of red blood cells by the bile salts was believed by E J Cohn to indicate the binding of these substances by albumin (2) The bile acids which are contained in the serum of patients with hepatic disease have been found in this laboratory to be largely non-dialyzable (3) These observations indicate that these substances are bound by serum proteins This binding to proteins is a factor which may influence the serum concentration and renal clearance of the bile acids, as well as their relationship to such other serum constituents as the lipids and bilirubin The present report is concerned with the binding of several bile acids by certain plasma protein fractions, as measured by the dialysis-equilibrium method, and with the effect of pH upon this reaction

TABLE I

Binding of bile acids by human plasma protein fractions

Plasma protein fraction* [Cohn (4)]	mM × 10⁻³ of bile acid bound by 100 mg. of protein			
	Deoxy cholic acid	Cholic acid	Glycodeoxy cholic acid	Glyco-cholic acid
I	0	0		
II	0	0		
III	1 5	0	1 6	0
IV-1	1 8	0	1 3	0
V	3 3	1 4	3 3	1 3

* Major components of these fractions are as follows I, fibrinogen, II, γ-globulin, III, β-globulins, IV-1, α-globulins, V, albumin

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[2] Fellow of the New York Heart Association

MATERIALS AND METHODS

Studies were made of the amount of bile acid bound by the different fractions of human serum protein isolated by Cohn's method (4) Ten ml of a 1 per cent solution of each fraction in buffer (0 15 M NaCl, 0 01 M Na phosphate, pH 7 6) was placed in a small cellophane dialysis sac and equilibrated at 5° C for 48 hours against 50 ml of a solution of the sodium salt of a cholanic acid dissolved in the same buffer At the same time 10 ml of buffer were equilibrated against 50 ml. of the solution of cholanic acid.[3]

After equilibration with buffer or serum protein, the bile acid concentration in the outer solution was determined spectrophotometrically (5, 6) on an aliquot of from 1 to 3 ml [4] The concentration of cholanic acid in the outer solution, which represents also the concentration of unbound bile acid within the sac, makes it possible to calculate the total quantity of unbound bile acid. By subtraction of unbound bile acid from the total bile

[3] The dialysis tubing was boiled three times and rinsed repeatedly with distilled water prior to use.

The equilibration was virtually complete after 24 hours Increasing the time beyond 48 hours produced no further change in the concentration of the cholanic acids in the outside phase. When only buffer was placed in the sac, the concentration of cholanic acid after equilibration was identical in the inner and outer solutions, and was equal to the value calculated from dilution. There was no evidence of adsorption by the membrane itself.

[4] In the spectrophotometric determination of bile acid possessing one hydroxyl group, the 65 per cent sulfuric acid reagent must be replaced by a 9 to 1 mixture of concentrated H_2SO_4 and glacial acetic acid, as was first shown in the case of lithocholic acid by Minibeck (7) Monohydroxy bile acids possessing a double bond in the ring system, however, exhibit a suitable ultraviolet absorption spectrum in 65 per cent H_2SO_4 after heating at 60° for 15 minutes

The contents of the sac were not analyzed since the presence of the protein decreases the exactness of the cholanic acid determinations

acid content of the system, the quantity of bile acid bound by the protein within the sac was calculated.

The Donnan effect was considered to be of negligible magnitude and was disregarded in the calculations.

Commercial samples of deoxycholic acid, hyodeoxycholic acid and cholic acid were employed in these studies. The following compounds were kindly supplied by Dr Erwin H. Mosbach Taurine and glycine conjugates of deoxycholic and cholic acids chenodeoxycholic acid lithocholic acid 7 hydroxy cholanic acid 3-hydroxy 12 keto cholanic acid 3, 7-dihydroxy 12 keto cholanic acid and the formyl derivatives of deoxycholic and cholic acids. 3 12-dihydroxy 7 keto cholanic acid was donated by Dr Norman A. Hulme of Sterling Winthrop Research Institute. 3-hydroxy 12 keto, Δ9-11 cholenic acid was given by Dr Karl Pfister of Merck Research Laboratories. The protein fractions of human plasma were generously donated by Dr J M. Ashworth of the American Red Cross through E. R. Squibb and Sons.

RESULTS

Binding of bile acids by various plasma proteins

The extent of binding of deoxycholic and cholic acids, by 100 mg of each of 5 plasma protein fractions is indicated by Table I The protein in a volume of 10 ml., was equilibrated against 12.7×10^{-3} mM of bile acid in a volume of 50 ml Among the plasma proteins albumin exhibits the greatest binding activity towards both of these bile acids The uptake of deoxycholic and glycodeoxycholic acids by Fractions III and IV 1 was approximately half that shown by albumin. Fibrinogen and gamma globulin did not bind either deoxycholic or cholic acid

Extent of binding by albumin of the series of cholanic acids

The chemical structure of the bile acid was found to influence the extent to which it is bound to human serum albumin

Ten ml of a 1 per cent human serum albumin solution (1.4×10^{-3} mM of albumin) was equilibrated with 50 ml of buffer containing 12.7×10^{-3} mM of a number of bile acids and their derivatives Table II lists the bile acids and derivatives studied, together with the number of moles of the bile acid bound by each mole of albumin under these conditions. The extent of binding decreases as the number of hydroxyl groups on the ring system is increased It was greatest for the bile acids with a single hydroxyl group and least for cholic acid which has three hydroxyl groups The position of the hydroxyl groups in the ring system has little influence upon the degree of binding Neither conjugation of the carboxyl group with glycine or taurine, nor covering up the hydroxyl groups by the formyl radicals greatly changed the extent of binding

The introduction of a keto group in either the 7 or 12 position of the ring system suppresses the affinity for albumin. In the only unsaturated compound tested (3-hydroxy, 12 keto Δ9 11 cho-

TABLE II

Binding of various cholanic acids by human serum albumin

Common name	Structure	Moles of cholanic acid bound by one mole of albumin
Monohydroxycholanic acids		
Lithocholic acid	3-OH cholanic acid	6.5
	7-OH cholanic acid	6 7
Dihydroxycholanic acids		
Deoxycholic acid	3 12 di-OH cholanic acid	2 3
Hyodeoxycholic acid	3 6 di-OH cholanic acid	3 2
Chenodeoxycholic acid	3 7 di-OH cholanic acid	3 0
Trihydroxycholanic acids		
Cholic acid	3 7 12 trihydroxy cholanic acid	94
Conjugated cholanic acids		
Glycodeoxycholic acid	3 12 di-OH cholanyl glycine	2 3
Taurodeoxycholic acid	3 12 di-OH cholanyl taurine	2.3
Glycocholic acid	3 7 12 tri-OH cholanyl glycine	92
Taurocholic acid	3 7 12 tri-OH cholanyl taurine	.59
Ketocholanic acids		
—	3-OH 12 keto cholanic acid	0*
—	3 7 di-OH 12 keto cholanic acid	0
—	3 12 dihydroxy 7 keto cholanic acid	0*
— (Unsaturated)	3-OH 12 keto Δ 9–11 cholenic acid	1 6
Formylated cholanic acids		
Diformyldeoxycholic acid	Diformyl 3 12 di hydroxy cholanic acid	3.2
Triformylcholic acid	Triformyl 3 7 12 tri hydroxy cholanic acid	1.2

* With larger amounts of albumin it is possible to demonstrate slight binding of these two cholanic acid derivatives by albumin

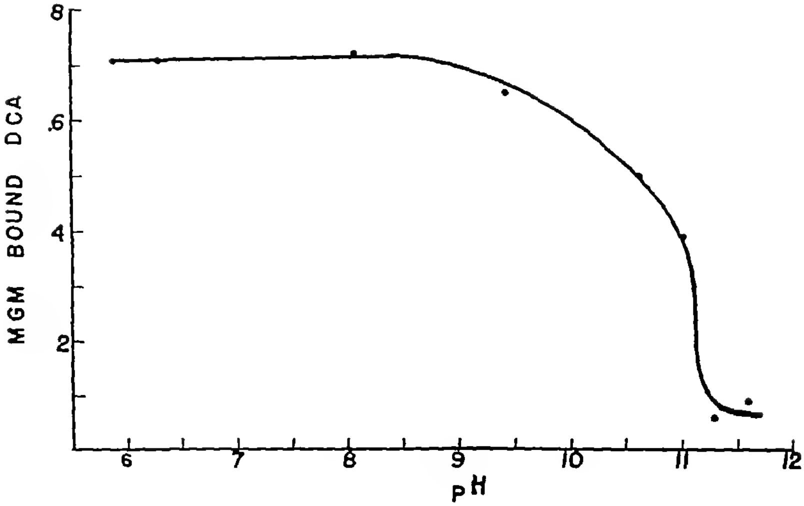

FIG 1 THE BINDING OF DEOXYCHOLIC ACID BY 50 MG OF HUMAN ALBUMIN AT VARIOUS pH'S

Five ml. of 1 per cent albumin solution in buffer was dialyzed against 2 5 mg of sodium deoxycholate in a volume of 25 ml. of buffer

lenic acid), the introduction of a double bond into the ring system increased the extent of binding with albumin

Effect of pH on binding reaction

The effect of hydrogen ion concentration upon the interaction with albumin was studied, in the case of deoxycholic acid, over a pH range of 5 9 to 11 6 (Figure 1) The reaction is indifferent to hydrogen ion concentration until the pH of 9 0 is reached Above pH 9 0 the affinity for albumin decreases rapidly, and is nearly absent above pH 11 0 Denaturation of albumin by alkaline pH was excluded by the finding that the albumin solutions, after the experiment had been completed, exhibited the usual uptake of deoxycholate at pH 7 6

Calculation of binding constants

It is apparent from Table II that each molecule of albumin can bind more than one molecule of cholanic acid The number of molecules of cholanic acid bound by one molecule of albumin is determined by the concentration of unbound cholanic acid The relationship between bound and unbound cholanic acid involves two parameters the dissociation constant of the albumin-bile acid complex (K) and the maximum binding capacity of the albumin molecule (n)

If it is assumed that in a molecule the size of albumin, the reactivity of a binding site is not markedly affected by the state of other binding sites, then the reaction can be treated as a simple bi-molecular reaction between the binding site and the cholanic acid

The equilibrium conditions of this reaction may be formulated as follows (8)

$$1/r = K/n \; 1/(A) + 1/n$$

where r is the ratio of moles of bound cholanic acid to moles of albumin, A is molar concentration of unbound cholanic acid, n is maximum moles of cholanic acid which can be bound by one mole of albumin, and K is dissociation constant of the albumin-cholanic acid complex It is apparent that a plot of 1/r as a function of 1/A should assume a linear form, the vertical intercept representing 1/n and the slope representing K/n By this method, the constants K and n may be calculated for each bile acid

Such data have been obtained for deoxycholic acid and cholic acid (Figure 2) Solutions of the sodium salt of each bile acid in buffer, were prepared with a bile acid concentration of $1\,27 \times 10^{-4}$, $2\,54 \times 10^{-4}$, $5\,08 \times 10^{-4}$, and $12\,70 \times 10^{-4}$ mM

per ml. Fifty ml. of each solution were equilibrated with 10 ml. of a 1 per cent solution of albumin in buffer. Measurement of unbound bile acid concentration in the outer solution after equilibration, and calculation of quantity of bile acid bound by the albumin within the sac, provided the data for the curves in Figure 1. From the intercept and slope of these curves, it may be calculated that for deoxycholic acid $K = 7.4 \times 10^{-4}$ and $n = 12$, for cholic acid, $K = 6.5 \times 10^{-4}$ and $n = 4$.

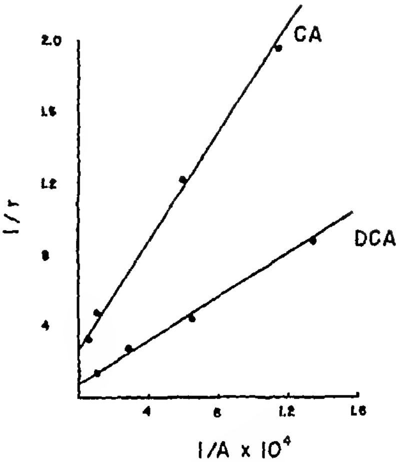

FIG. 2. BINDING OF DEOXYCHOLIC ACID (DCA) AND CHOLIC ACID (CA) BY HUMAN ALBUMIN AS A FUNCTION OF CONCENTRATION OF THE UNBOUND ACID

DISCUSSION

The binding of the cholanic acid series of compounds by serum albumin might have been predicted from knowledge of the interaction of serum albumin with long chain fatty acids and with a variety of other organic anions (9). Serum albumin has been considered to be unique among the plasma protein fractions in its ability to bind organic anions (10). The present data confirm the preeminence of albumin in this regard, but indicate that the lipoprotein-containing Fractions III and IV 1 also interact with the cholanic acids although to a lesser extent than does albumin.

The pH effect, namely the suppression of binding above pH 9, is compatible with the existence of an electrostatic bond between the positively charged ε-amino group of lysine (pK 9.3) and the negatively charged carboxylate group of the cholanic acid, as the primary force responsible for the binding. This finding parallels that of Klotz and Walker in the binding of methyl orange by bovine serum albumin (11).

However, the existence of secondary forces between albumin and the bile acid is indicated by the variation in extent of binding among closely related cholanic acids (Table II). The data indicate that the polarization of the cholanic acid ring system by the successive introduction of hydroxyl groups suppresses the affinity for albumin.

There are three times as many sites in albumin available for binding deoxycholic acid, a dihydroxy compound, as are available for binding cholic acid, which possesses 3 hydroxy groups. These observations are consistent with the postulated role of van der Waal forces acting upon the non polar region of the smaller molecule, in determining the affinity of the substance for albumin (10). The marked reduction of affinity for albumin which results from the presence of a keto group in the ring system likewise appears to be caused by changes in the polarity of the ring system.

The cyclopentano-perhydrophenanthrene ring system of the bile acids is also found in such biologically important substances as cholesterol, steroid hormones and cardiac glycosides, which differ from one another in the structure of the ring system and in the nature of substituent groups upon the ring. Study of how the interaction of cholanic acids with albumin is modified by changes in the structure of the cholanic acids may yield information of interest. The testing of additional cholanic acid derivatives will provide further information on the relationship between molecular structure and affinity for the various plasma proteins.

SUMMARY

The binding of bile acids and their derivatives by the protein fractions of human plasma has been studied by the dialysis-equilibrium method. Albumin exhibits the greatest binding activity to-

wards these compounds The lipoprotein-containing globulins, Cohn Fractions III and IV-1, bind approximately half as much deoxycholic acid and cholic acid as does albumin γ-globulin and fibrinogen do not interact with the bile acids

The affinity for albumin is reduced by the introduction of polar groups into the steroid nucleus Thus the extent of binding decreases in the order monohydroxy > dihydroxy > trihydroxy cholanic acid Binding of keto-cholanic acids to albumin could not be detected

The effect of pH upon the binding reaction suggests that the primary attraction between albumin and the cholanic acids is an electrostatic bond between the positively-charged lysine side chains of the former and the negatively-charged carboxyl groups of the latter

The binding constants of albumin with two bile acids were calculated

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INHIBITORY EFFECT OF CHLORPROMAZINE UPON THE ADRENAL CORTICAL RESPONSE TO INSULIN HYPOGLYCEMIA IN MAN [1]

By NICHOLAS P CHRISTY [2] DONALD LONGSON [3] WILLIAM A. HORWITZ, AND MARY M. KNIGHT

(From the Departments of Medicine and Psychiatry the College of Physicians and Surgeons the Presbyterian Hospital and the New York State Psychiatric Institute New York N Y)

(Submitted for publication October 12, 1956 accepted December 13 1956)

The large number of recent publications concerning the inter relationships between the central nervous system and the anterior pituitary testifies to the intense interest in this subject (2, 3) Particular attention has been devoted to the effects of hypothalamic stimulation or injury upon pituitary ACTH release and adrenal cortical response. The work of deGroot and Harris (4) Hume and Wittenstein (5), Porter (6), McCann (7) and many others has indicated the importance of hypothalamic integrity in the normal release of adrenocorticotropin from the pituitary in response to a stimulus

Another approach to the problem of neural control of the pituitary has been made through the use of central nervous system-depressant drugs such as morphine (8), which reportedly exerts an inhibitory influence upon pituitary release of corticotropin in response to acute stressing procedures Among the drugs so used is the phenothiazine derivative 10-(γ-dimethylaminopropyl) 2 chlorophenothiazine ('largactil," chlorpromazine) A major site of action of this agent has been thought to be the hypothalamus (9) Some weight was lent to this supposition by the finding of Wase, Christensen, and Polley that chlorpromazine labeled with S^{35} was accumulated in the hypothalamus in concentrations exceeding those attained in other areas of the brain (10)

Reports of the effect of this drug upon the adrenal response (and by inference, upon the hypothalamic pituitary adrenal response) to various stresses have described conflicting findings Using rat adrenal ascorbic acid-depletion as the index of adrenal response Aron (11) Hamburger (12) Ohler, Sevy, and Weiner (13), and Olling and deWied (14) have independently shown that chlorpromazine apparently blocked adrenal ascorbic acid depletion after operative shock. Holzbauer and Vogt (15) found that the drug failed to inhibit this response, and Cheymol deLeeuw and Oger (16) were able to show only a partial interference Additional difficulties arose from the work of Georges and Cahn (17) who observed that chlorpromazine could itself produce eosinopenia in rats of Egdahl, Richards, and Hume (18) who demonstrated elevations of cortisol levels in adrenal venous blood of intact dogs after intravenous chlorpromazine administration, of Hamburger (12) who showed moderate ascorbic acid depletion after chlorpromazine alone and of Harwood (19) who reported rises in plasma 17-hydroxycorticosteroid values in monkeys given the drug

In the face of these conflicting data, the following study was undertaken in an attempt to clarify the effects of chlorpromazine on the adrenocortical response of human subjects to acute stimuli

METHODS AND MATERIALS

1. *Experimental procedure* The stimulus employed in this study was insulin coma as used in the treatment of patients with schizophrenia. The index of pituitary adrenal response was the rise in plasma 17-OH-corticosteroid levels. Such rises have been reported to occur even after mild insulin hypoglycemia (20) Plasma steroid responses to insulin in schizophrenic patients were shown to be entirely comparable to the normal by Bliss, Migeon, Branch, and Samuels (21)

The procedure was as follows. Nine patients, seven females and two males aged 15 to 44 who were undergoing insulin coma treatment for schizophrenia were selected. Control blood samples for plasma corticosteroid

[1] Supported in part by a grant to Dr Joseph W Jailer from the National Institute of Arthritis and Metabolic Diseases (A 195[C]) These data were presented in preliminary form at the 38th Annual Meeting of the Endocrine Society June, 1956 (1)

[2] John and Mary R. Markle Scholar in Medical Science.

[3] Dickenson Traveling Fellow University of Manchester England.

determinations were drawn at 6 30 a m Standard insulin was then administered subcutaneously in doses of 45 to 670 units Two additional blood samples were then drawn, one at the beginning of coma, and the second just before its termination (*i e*, at 3 and 4 hours after insulin)

Four to 21 days later the same procedure was repeated after pre-treatment at 4 30 a.m and at 6 30 a.m. with 50 to 150 mg of chlorpromazine hydrochloride given orally, for total doses of 100 to 300 mg This timing was selected in an effort to attain maximal blood levels of the drug at the inception and at the height of coma (9)

Three to 17 days after the chlorpromazine experiment was performed in a given patient, the procedure was repeated without chlorpromazine.

In eight of the nine patients, blood specimens for glucose determination were obtained at 0 time (6 30 a.m) and 3 hours after insulin administration

2. *Effect of chlorpromazine upon response of the adrenal cortex to exogenous ACTH* In order to rule out the possibility that chlorpromazine might exert an effect upon adrenal cortical response to ACTH, patients treated with the drug in large doses (400 to 1,000 mg per day) for periods of 25 to 70 days were subjected to standardized intravenous ACTH tests in a manner previously described (22), the index of response again being the rise in plasma 17-OH-corticosteroid levels

In addition, three patients received chlorpromazine acutely, with a dose schedule like that employed in the insulin coma studies, and then were subjected to intravenous ACTH tests

3 *Analytical methods* Levels of plasma 17,21 dihydroxy-20-ketosteroids were estimated by the Silber-Porter method (23), as modified in this laboratory (24), and more recently, by a procedure which incorporated the essential features of the changes made by Peterson, Wyngaarden, Guerra, Brodie, and Bunim (25) and which eliminates the need for adding cortisol to the unknowns

Blood glucose determinations were made according to the method of Benedict (26)

For more specific measurement of plasma 17-OH-corticosteroids, paper chromatography was resorted to This was deemed necessary because it was noticed as the study progressed that a pink color formed in the blank tubes of plasma samples taken from patients who had received large doses of chlorpromazine. *In vitro* studies revealed that this chromogen was due to chlorpromazine and that it also interfered with optimal development of Porter-Silber chromogen, *i.e*, with the development of the color reaction between cortisol and phenylhydrazine sulfuric acid. Readings were reduced, at most, to values 15 per cent below control levels in *in vitro* experiments in which chlorpromazine in amounts equivalent to 250 micrograms per 100 ml. plasma was added to known amounts of cortisol.[4] Attempts to separate chlorpromazine from cortisol *in vitro* and *in vivo* by washing extracts with 1 N sulfuric acid (9) and by means of column chromatography (18) were not successful.

Separation was achieved by paper chromatography Plasma was extracted with ethyl acetate, washed with base, subjected to hexane methanol partition, and the dried extract chromatographed in a formamide chloroform system as described by Burton, Zaffaroni, and Keutmann (27) The cortisol region was detected by ultraviolet light absorption and mobility in comparison with a simultaneously chromatographed reference standard of steroid. Quantitation of cortisol (and further confirmation of its probable identity) was carried out after methanol elution from paper by the colorimetric methods of Porter and Silber (28), and (in one instance) Gornall and Macdonald (29) In each case, blanks (sulfuric acid and ethanol without phenylhydrazine or 2,4-dinitrophenylhydrazine) were run on the eluate of the cortisol region of the paper chromatogram. The fact that no pink color was detectable in any of these blanks was taken as evidence that the chlorpromazine had been separated from the steroid. In the 11 samples tested by the Porter-Silber method (28), unknowns formed the usual yellow color with an absorption maximum at 410 millimicra. Corresponding blanks did not give inordinately high readings (4 to 25 per cent of the readings of unknowns) either in the chlorpromazine-treated specimens or in those which were untreated.

Recoveries of known amounts of cortisol ranged from 35 to 75 per cent by this technic. *In vitro* addition of chlorpromazine in amounts as large as 100 micrograms did not alter recoveries of cortisol

Plasma samples which were subjected to these extraction and chromatographic procedures were obtained from 12 additional patients with insulin coma. Large samples of blood were drawn at 3 and at 4 hours, and the two specimens from a given patient pooled together Quantitation of post-insulin cortisol levels in the group receiving only insulin was compared with quantitation of the steroid in the group receiving insulin plus chlorpromazine.

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[4] No reliable quantitative data are yet available concerning the plasma levels of chlorpromazine attained in human subjects given the usual doses of the drug (9) The figure, 250 micrograms per 100 ml plasma, was arrived at after trial and error as a quantity which produced the pink chromogen to a degree greater than that encountered in plasma specimens obtained from the chlorpromazine-treated subjects Plasma concentrations in the patients were therefore assumed to be less than 250 micrograms per 100 ml. The validity of that assumption appears to be borne out by data presented in a study published since the preparation of this report (Salzman, N P, and Brodie, B B, Physiological disposition and fate of chlorpromazine and a method for its estimation in biological material. J Pharmacol & Exper Therap, 1956, 118, 46) Dogs receiving 20 mg per kg of chlorpromazine intravenously attained plasma levels of 70 to 260 micrograms per 100 ml during the period, 0.5 to 3 hours after administration. The doses given to the subjects in the present study were of the order of 1 5 to 4 5 mg per kg

TABLE I

Response of plasma 17-OH-corticosteroid levels to insulin hypoglycemia
Initial control experiments

nt	Insulin (units)	Plasma 17-OH-corticosteroids (micrograms %) Control	3 Hours	4 Hours
J S	420	23	37	43
M K.	390	21	26	39
J Sc.	500	27	41	53
S L.	160	19	33	34
R. M	270	19	48	46
S. R.	50	26	33	45
J D	630	20	29	30
I S.	100	14	27	40
H. M	320	22	33	44
Average	316	22	34	42

TABLE II

Effect of chlorpromazine upon plasma 17-OH-corticosteroid response to insulin hypoglycemia

Patient	Insulin (mg)	Chlor promazine (Total dose)	Plasma 17-OH-cortico-steroids (micrograms per 100 ml.) Control	3 Hours	4 Hours
J S	400	300	30	23	27
M K.	410	200	27	10	23
J Sc.	170	200	23	14	7
S L.	160	100	27	14	13
R. M	300	300	22	0	27
S R.	45	200	15	8	8
J D	630	200	11	6	19
I S	120	200*	23	15	22
H M	300	300	4	8	11
Average	281		20	11	17

* A dose of 150 mg chlorpromazine failed to suppress adrenocortical response to insulin hypoglycemia in this patient.

RESULTS

Response of plasma 17 OH-corticosteroid levels to insulin coma

Effects of insulin coma upon plasma 17-OH corticosteroid values are summarized in Table I It will be seen that resting levels fell within the normal range (4 to 28 micrograms per 100 ml. [24]), and at 3 and at 4 hours after insulin there was a consistent rise to mean levels of 34 and 42 micrograms per 100 ml respectively These levels were above the normal range and were roughly comparable to levels attained by normal individuals following intravenous administration of 25 i u. ACTH (22) There appeared to be no correlation between the magnitude of plasma 17-OH-corticosteroid rise and the size of the dose of insulin.[5]

Effect of chlorpromazine upon plasma 17 OH-corticosteroid response to insulin coma

Table II shows the results in the same patients pre-treated with chlorpromazine It is apparent that the resting levels of plasma 17 OH-corticosteroids were comparable to those found in the control experiments (mean levels 20 and 22 micrograms per 100 ml. respectively) In contrast to the control responses, 17 OH-corticosteroid levels during coma did not rise but remained within normal limits The trend of values was reminiscent of the normal diurnal variation of plasma 17-OH-corticosteroid levels observed by Bliss, Sandberg Nelson, and Eik Nes (30) The duration and depth of coma did not appear to differ from the coma seen during the control experiments without chlorpromazine. The only clinical differences noted were diminution of salivation and sweating in the chlorpromazine-treated group No differences in depression of blood pressure were found.

In the repeat control experiments plasma 17-OH-corticosteroid levels rose as they had in the initial controls in six of eight subjects (Table III) The data from control and chlorpromazine experiments in four of the patients are presented graphically in Figure 1

In two additional patients chlorpromazine did not clearly inhibit the adrenal cortical response to insulin. Since the control rises in plasma 17 OH corticosteroid levels were also equivocal, it was thought reasonable to exclude the findings in these cases from the data presented.

As shown in Table IV depressions of blood glucose in the control and chlorpromazine studies did not differ appreciably

Chromotographic studies of plasma 17 OH corticosteroids during insulin coma

Table V summarizes the results of chromatographic fractionation and quantitation of plasma cortisol levels In the Table are compared the

[5] This dose was arrived at by purely empirical means, being the dose required to produce satisfactory coma which was not unduly prolonged. The required dose was extremely variable from patient to patient, and tended in most cases to become progressively smaller during the course of the series of coma treatments.

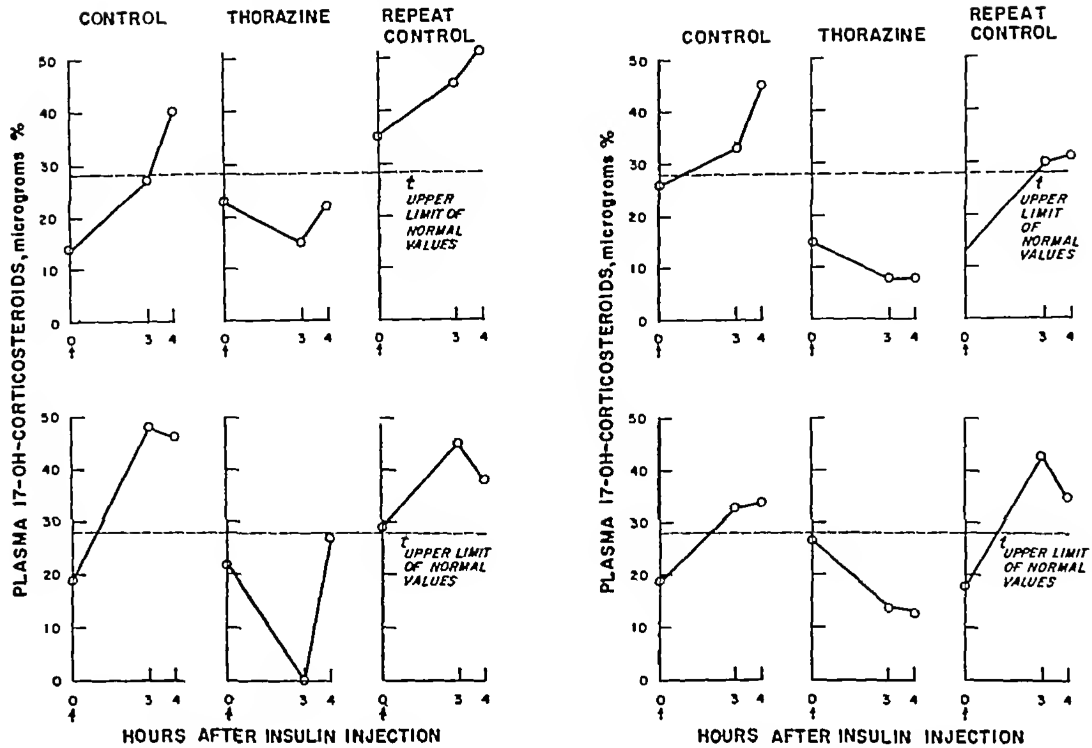

Fig 1 Effect of Chlorpromazine upon Adrenocortical Response to Insulin Hypoglycemia

Arrows indicate time of insulin injection Data obtained from patients I. S , S R., R. M , and S L. (reading left to right)

amounts of cortisol detected during insulin coma with and without chlorpromazine pre-treatment Without chlorpromazine, the mean value for plasma cortisol after chromatography was 18 0 micrograms per 100 ml (without chromatography, 34 6 micrograms per 100 ml , "recovery," 52 per cent) In the chlorpromazine-treated subjects, the average value was 8.7 micrograms per 100 ml (without chromatography, 16 7 micrograms per 100 ml , "recovery," 52 per cent) The difference between the means of the two groups was statistically significant ($P < 0\ 01$) The fact that about the same proportion of cortisol was recovered in control and chlorpromazine experiments after chromatography lessened the suspicion that the difference in cortisol levels could be accounted for by an adverse effect of chlorpromazine itself upon steroid recovery This already seemed unlikely from *in vitro* studies (cf Section 3, *Methods and Materials*)

Effect of chlorpromazine upon plasma 17-OH-corticosteroid response to exogenous ACTH

Table VI shows the effects of long- and short-term chlorpromazine administration upon the response of the adrenal cortex to exogenous ACTH It is obvious that only one of the patients (A T) showed a very slightly reduced response Despite the inhibitory effect of the drug upon plasma corticosteroid response to insulin, it did not bring about a suppression of ACTH-responsiveness of the adrenal cortex as does prolonged steroid administration (31) Similarly, acute administration of the drug, given according to the dose schedule used in the insulin studies, had no suppressive effect upon the response to ACTH

DISCUSSION

The data presented confirm the efficacy of insulin hypoglycemia as a stimulus to the adrenal cortex in man (20) The results also indicate

TABLE III

Response of plasma 17-OH-corticosteroid levels to insulin hypoglycemia

Repeat control experiments

Patient	Insulin (*units*)	Plasma 17-OH-corticosteroids (*micrograms %*) Control	3 Hours	4 Hours
J S				
M K.	400	37	48	48
J Sc.*	190	26	27	38
S. L.	160	18	43	35
R. M	200	29	45	38
S. R	45	14	30	31
J D	670	29	31	34
I S	120	35	45	51
H M	110	22	17	25
Average	237†	26	36	38

* This patient received 100 mg phenobarbital prior to experiment.

† Note that average dosage of insulin is less than in the chlorpromazine experiment (Table II) yet adrenocortical response still occurs in a manner comparable to initial controls (Table I)

that chlorpromazine can inhibit the adrenocortical reaction to this stimulus The inhibition of plasma 17-OH-corticosteroid rise after insulin which was caused by chlorpromazine does not seem to be an artifact. Chromatographic separation and quantitation of steroid revealed that cortisol in plasma of the chlorpromazine-treated subject was reduced by about 50 per cent in comparison with the control. This was essentially the same degree of reduction found in unchromatographed plasma specimens

TABLE IV

Lack of influence of chlorpromazine upon blood glucose response to insulin hypoglycemia

Patient*	Blood glucose (*mg per 100 ml*) Without chlorpromazine: Control	Without chlorpromazine: 3 Hours	With chlorpromazine: Control	With chlorpromazine: 3 Hours
M K			70	30
J Sc.	97	42	101	39
S L.			87	29
R. M	97	30	103	31
S R.	82	33	94	42
J D			103	31
I S			69	19
H M	94	32	97	34
Average	93	34	91	32

* In all but one of the patients in whom blood glucose responses were recorded both with and without chlorpromazine, insulin doses were comparable.

TABLE V

Results of chromatographic analysis of cortisol present in plasma of patients during insulin coma with and without chlorpromazine pretreatment

Specimen	17-OH-corticosteroids (*micrograms per 100 ml*) Without chlorpromazine: Before chromatography	Without chlorpromazine: After chromatography*	With chlorpromazine: Before chromatography	With chlorpromazine: After chromatography*
1	32 5	21 0		
2		16 4		
3	38.2	12 9		
4	34 9	11 4		
5	36 0	26 0		
6	31 2	20 0		
7			15 2	7 6
8			15.3	9 8
9			20 9	11 2
10			12.3	10 0
11			21 6	8 0
12			15 0	5 8
Average	34 6	18.0†	16 7	8 7†
S D		5 5†		1 9†
Cortisol recovered after chromatography (Average, %)		52		52

* Values after chromatography have not been corrected for losses.

† T test shows statistically significant difference between the mean values ($P < 0.01$)

The results appear to support those of certain previous investigations in animals (11–14) The absence of definite blockade of adrenal cortical response reported by other workers (15–17) may perhaps be explained by differences in dosage and

TABLE VI

Lack of inhibitory effect of chlorpromazine upon response of plasma 17-OH-corticosteroid levels to a standard intravenous ACTH test

Patient	Chlorpromazine dose/day (*mg*)	Duration of treatment (*days*)	Plasma 17-OH-corticosteroids (*micrograms per 100 ml.*) Before ACTH	After ACTH
A E	400	25	25	59
A. T	600–1000	30	17	33
H G	1000	30	41	63
R. D	400–1000	70	19	47
J D	200	*	12	37
S. R.	200	*	11	37
M G	200	*	11	66
Normal range (31)			4–23	35–59

* Chlorpromazine administered in these three patients according to the dose schedule employed in the insulin coma experiments.

in timing of administration of the drug in relation to the stimulus imposed, as Hamburger has suggested (12) The findings presented here and earlier (11–14) are not necessarily incompatible with the adrenocortical-stimulating property of chlorpromazine demonstrated by Hamburger (12), by Egdahl, Richards, and Hume (18), and by Harwood (19) The factors of speed and route of administration may play a role, and as Harris has pointed out, chlorpromazine may be to some extent a toxic compound like other "blocking agents" which have been used in similar experiments and which themselves cause corticotropin release (2)

This experiment does not elucidate the mechanism of the demonstrated blocking action of chlorpromazine *It seems clear from* the *findings* presented here and from the work of Olling and deWied (14) that the drug does not interfere with the action of adrenocorticotropin upon the adrenal cortex itself There is no experimental precedent to suggest that this agent can cause an increased rate of disappearance of cortisol from plasma, or accelerated hepatic reduction and conjugation of cortisol To date, an increased rate of cortisol disposal has been demonstrated only in hyperthyroidism (25)

In trying to visualize the mechanism of the blockade, one is tempted to assume that the site of action of chlorpromazine in inhibiting adrenocortical response (and by inference, pituitary corticotropin release) might be the hypothalamus Some support for this assumption might be derived from the known suppressive effects of chlorpromazine upon the hypothalamus (9), from the moderately selective accumulation of chlorpromazine in hypothalamic structures (10), and from the knowledge that experimental damage to certain hypothalamic nuclei may result in inhibition of ACTH release in response to a stimulus (4–7) However, such a chain of reasoning must be viewed as entirely speculative, and it should be realized that the data presented in this report, which might be construed as supporting such a concept, constitute only indirect and circumstantial evidence

The fact that relatively prolonged administration of chlorpromazine failed to suppress plasma 17-OH-corticosteroid response to exogenous ACTH (in contrast to long-term steroid administration [31]) is not necessarily incompatible with the inhibitory effect of the drug upon adrenal cortical response to an acute stimulus The two apparently contradictory findings may perhaps be reconciled by Harris' concept of a dual control of the central nervous system over corticotropin release, one mechanism operating under quiescent conditions, the other under conditions of "stress" (2) Such an explanation is again speculative, and it is emphasized that the above discrepancy cannot be satisfactorily accounted for on the basis of present information

Finally, it is perhaps of some importance that, however produced, blockade of adrenal cortical response to the rather severe stress of insulin hypoglycemia did not result in any clinical symptoms suggesting adrenal cortical insufficiency *Similar observations were made when adrenal* cortical response to typhoid vaccine administration was prevented by blocking the pyrogenic reaction with aminopyrine (32) These findings may be interpreted as supporting the concept of the "permissive" role of the adrenal cortex in homeostasis as stated by Ingle (33), and again raise a question as to the advantage for the organism of large increases in adrenal cortical activity in response to acute stimuli

SUMMARY

1 Insulin coma was again shown to cause adrenal cortical response in man, as measured by rises in plasma 17-OH-corticosteroid levels

2 Chlorpromazine usually inhibited this adrenal cortical response to insulin hypoglycemia

3 Chlorpromazine had no inhibitory effect upon the response of the human adrenal cortex to administered adrenocorticotropin

ACKNOWLEDGMENTS

The authors wish to express their thanks to Dr Joseph W Jailer, in whose laboratory the work was performed, for his advice and encouragement. Grateful acknowledgment is also made to Mr Milan Jackson for his invaluable help, and to Miss Elsie Ewen for technical assistance. Thanks are due to the Upjohn Company and to Organon, Inc, for the generous provision of supplies

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RENAL REABSORPTION OF PHOSPHATE IN NORMAL HUMAN SUBJECTS AND IN PATIENTS WITH PARATHYROID DISEASE

By DAVID D THOMPSON [1] AND HOWARD H. HIATT [2]

(From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U S Department of Health, Education and Welfare, Bethesda, Md)

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A number of studies in the dog and in man (1–6) have demonstrated a maximal renal tubular reabsorptive capacity for phosphate (TmP [3]) However, some investigators have denied that the reabsorption of phosphate conforms to Tm characteristics (7–9) These workers have suggested that phosphate reabsorption is conditioned by the rate of phosphate filtration such that a fairly constant proportion of the filtered phosphate is reabsorbed In view of the conflicting evidence and the relative paucity of phosphate titration studies on man, experiments were performed in order to obtain information on the mechanism of phosphate reabsorption, the constancy of reabsorption in any one individual, and its variability in a group of subjects The data were required for subsequent investigations designed to evaluate the influence of several factors on renal phosphate transport The studies were performed on normal individuals and in patients with parathyroid disease

It was found that in any given experiment a TmP was demonstrable However, the variability in TmP seen in most of our normal subjects between consecutive periods of a single experiment and between experiments generally exceeded the variability reported for glucose Tm and for PAH Tm Furthermore, the range of values for TmP within a group of normal individuals is so large that an average value has little meaning Fluctuations in TmP in our subjects could not be consistently correlated with the level of calcium or phosphate in the diet

[1] Present address Department of Physiology, Cornell University Medical College, New York 21, N Y

[2] Present address Department of Medicine, Harvard Medical School and Beth Israel Hospital, Boston, Mass

[3] The following abbreviations will be used in this paper P, inorganic phosphate, C_{In}, inulin clearance, GFR, glomerular filtration rate, RPF, effective renal plasma flow, Tm, maximal rate of renal tubular reabsorption Ca, calcium, K, potassium, PAH, para-amino hippurate.

METHODS

Subjects Studies were carried out on ten normal subjects, three patients with post-thyroidectomy hypoparathyroidism, and one individual with hyperparathyroidism. The normal individuals included five male and three female volunteers aged 20 to 27 years, one 17-year-old male (C. H) convalescing from a hip injury, but ambulatory during most of the studies, and a 26-year-old female (W H.) hospitalized with a diagnosis of hypoparathyroidism, but found to have normal parathyroid function. The hypoparathyroid patients, a 37-year-old male and two females, aged 42 and 52, had not been treated previously with any medication other than calcium salts The manifestations of hypoparathyroidism were severe in the male and mild in the females The hyperparathyroid subject, a 72-year-old male, was subsequently cured by the removal of a parathyroid adenoma.

All subjects were hospitalized on a metabolism ward, and during many of the studies they received constant diets of calculated calcium and phosphate content. There were no restrictions on the patients' activities

Design of experiments Studies were begun about 9 a.m with the patient fasted for 12 to 16 hours One-half hour prior to the start of each study the subject ingested a liter of water and thereafter drank 200 ml. of wa-

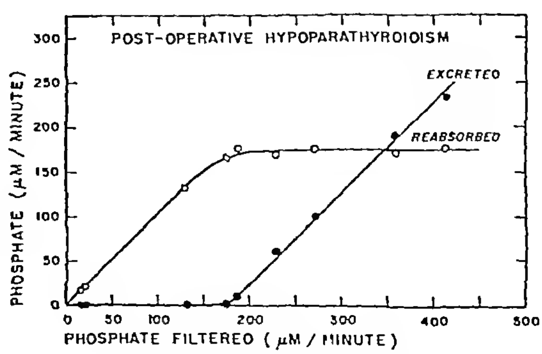

FIG 1 THE QUANTITIES OF PHOSPHATE FILTERED, EXCRETED, AND REABSORBED DURING INTRAVENOUS PHOSPHATE LOADING IN A HYPOPARATHYROID SUBJECT

TABLE I

Effect of elevation of plasma inorganic phosphate on the quantity of phosphate filtered excreted and reabsorbed *

Total concurrent time *min.*	Urine flow *ml./min*	C_{In} *ml./min.*	C_{PAH} *ml./min*	Plasma P *µM/ml.*	Urine P *µM/ml*	Phosphate Filtered *µM/min.*	Phosphate Excreted *µM/min.*	Phosphate Reabsorbed *µM/min.*	P reab / P filt.
35–39	Prime containing inulin and PAH given								
39	Infusion I containing inulin and PAH begun at 3.2 ml per min								
90–101	11.2	127	525	0 91	0 02	116	0.2	116	1 00
101–116	11.3	130	541	0 93	0 02	121	0 2	121	1 00
118	Infusion II begun sodium phosphate at 0.55 mM per min. plus inulin and PAH								
148–180	4.28	133	526	1 95	17.5	260	75	185	0 71
180–214	2.92	134	504	2.82	48 0	378	135	243	0 64
214–241	7.39	142	560	3.25	29.2	461	216	245	0.53
241–267	5 70	138	509	3 49	44 5	481	254	227	0 47

* Patient C. H., normal male subject, age 17

ter every 20 to 30 minutes to maintain a water diuresis. Female subjects were catheterized with a six hole rubber catheter which remained in place throughout the experiment. In the early studies the male subjects were also catheterized. However it was found that these individuals were able to void on command hence, catheterization was not performed in later experiments. After a sample of venous blood and one of urine were obtained for determination of inulinoid blank, a priming dose of inulin and PAH calculated to provide plasma levels of 20 and 2 mg per cent, respectively was given over a period of 3 to 5 minutes. Constant infusion of these substances dissolved in physiological saline solution was maintained by a pump Thirty to sixty minutes after the sustaining infusion was begun, timed urine collection periods were started. The period varied in duration from 20 to 40 minutes, and at the midpoint of each, blood was drawn from an antecubital vein into a heparinized syringe, immediately centrifuged, and the plasma separated. To close each period urine was collected by spontaneous voiding or through the catheter followed by irrigation with 20 to 60 ml. of distilled water and about 30 ml. of air

After two to four periods the infusion was changed to one containing buffered sodium phosphate, pH 7 40 in addition to inulin and PAH. A sufficient volume of a 0.5 M phosphate solution was incorporated in the infusion to provide for the delivery of 0 4 to 0.8 mM P per minute. Such an infusion was continued for sixty minutes before urine collections were again begun. This period was sufficient to allow elevation of the plasma P to the desired levels and to assure a relatively slowly increasing plasma P concentration during the collection periods. Studies were carried out for two to four periods at the elevated plasma P levels. In some experiments there followed a third infusion designed to elevate further the plasma P

Dietary intake The low intermediate and high calcium diets contained 130 800 and 1500 mg., respectively daily A low P intake was achieved by the administration of a diet containing 600 mg of P daily and, in several studies by administering in addition 35 ml. of aluminum carbonate gel, one hour after each meal and at bed time. The intermediate P diet contained 1,200 mg., and the high P diets 1800 to 3 000 mg P In several experiments variations in Ca or P intake without changes in other dietary constituents were studied by the administration of a constant low Ca-low P diet supplemented at meal time with calcium lactate or 10 per cent phosphoric acid. The patient was placed on a given dietary regimen at least three days and often more than a week prior to the renal function studies.

Chemical methods Inulin was determined in plasma and urine by the method of Schreiner (10) PAH by the method of Smith, Finkelstein, Aliminosa, Crawford, and Graber (11) Inorganic P by the method of Fiske and Subbarow (12) Plasma Ca was determined by the method of Clark and Collip (13) and urine Ca by the method of Shohl and Pedley (14) Plasma K was determined on a flame photometer utilizing an internal standard.

Calculations The clearances of inulin and PAH were taken as measures of GFR and RPF respectively Filtered P was calculated as the product of the inulin clearance and the plasma P Excreted phosphate was calculated as the product of urine P concentration and urine flow The difference between filtered and excreted P was considered to be the reabsorbed P

RESULTS

In thirteen studies on four normal and one hypoparathyroid subjects P reabsorption was determined at endogenous plasma P levels and following stepwise elevations of the plasma P In all instances a maximal rate of phosphate reabsorption was reached, and further elevation of plasma P resulted in no further increase in the rate of reabsorption This result is shown for one normal subject in Table I Following two

and Casey in a paper (2) to which the reader can be referred for further details It may suffice to say that in the summer and fall of 1941, 871 cases of poliomyelitis were reported in the state of Alabama, and of these, 125 occurred in Walker County (population 67,000) One of the areas with the highest case rate in this county was the small town of Cordova (population 1670) Here, within an area of 15 to 20 acres, 12 cases of poliomyelitis occurred among a total population of 181 persons All of these cases occurred in children and all remained in the community while sick It is probable that there were even more cases as will be seen from the diagram in Fig 4 designating febrile illnesses among the 64 juveniles during the period from early June unil late September Late in this epidemic, stool specimens were collected from 176 individuals (almost the entire group within the designated epidemic area) and tested by Wenner and Casey None of the 112 tested adults yielded a positive test for the virus of poliomyelitis but 3 of the 64 children, whose stools were collected

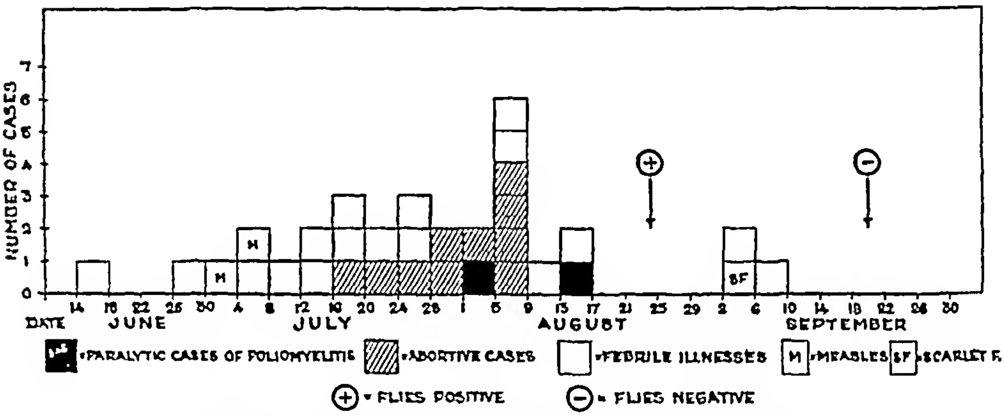

FIG 4 The epidemic within one district of Cordova, Alabama During the period indicated at the base of the chart, the juvenile population numbered 64

between Aug 15 and Oct. 6 were positive It is safe to say then that 3 or more carriers were present in this epidemic area at the time the first collection of flies was made (Aug 24) and only one known carrier was present at the time the second collection was made, Sept. 19

On Aug 19, which was the time of our first visit to this area,—a site for fly trapping was selected It was located in the heart of the epidemic area, back of a crowded group of ramshackle houses and adjacent to several privies The occupants of at least three houses used one of these privies and in two of these houses 3 cases of poliomyelitis had occurred with onsets between July 25 and Aug 6 There was a reasonable chance therefore (although this is not proven) that virus was being deposited within this privy into which flies were freely passing The fly trap was set a few feet from this privy (see Fig 5), and the catch obtained on Aug 24 was shipped in dry ice by air mail to New Haven where it was found positive for the virus Another catch similarly collected and sent on Sept 19, proved negative

In summary then, there is little doubt that at the time the first specimen of flies was collected, there were probably several intestinal carriers in the com-

munity, although a carrier survey made 1 month later, revealed only 3. There was also some reason to believe that a potential source of virus existed in this community during the latter half of August, in a privy to which flies in sample A 1 might have been exposed.

Sample NB-1. New Brunswick, Canada.[4]—During the summer of 1941 and again in the early spring of 1942 poliomyelitis appeared in epidemic form in the Province of New Brunswick, Canada. More than 200 scattered cases had been reported by mid September, 1941.

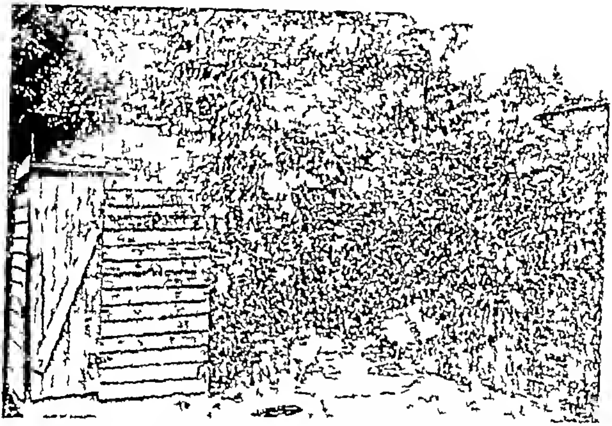

FIG. 5. The site where flies carrying poliomyelitis virus were trapped at Cordova, Alabama. At the time (Aug. 19) at which the fly trap was first set (within the automobile tire in the foreground) several children used this privy, including at least 3 who were recently convalescent from poliomyelitis.

On Sept. 21 and 22 flies were collected by Dr. J. M. Cameron, District Medical Health Officer at Minto, New Brunswick, and these were shipped to us in dry ice by air express. Fly trapping was accomplished according to the technique described in the preceding paper and the samples were obtained from the premises of two households: (a) those of A.L., age 3—a paralytic case with onset on Sept. 7 whose home was described as dirty with many flies, and (b) the home of H.W. Jr., age 14, a non-paralytic case with onset Sept. 15. This patient was not sent to the hospital but

[4] We are indebted to Dr. Arnold Branch of the Bureau of Laboratories, Department of Health, Saint John, to Dr. C. W. MacMillan, Chief Medical Officer of New Brunswick, and to Dr. J. M. Cameron of Fredericton, New Brunswick, Canada, for collecting these samples.

... samples were pooled for testing and virus was isolated ...

... h regard to some of the environmental circumstances are ... that in one of these homes a patient was present who was in the ...

... Which Only Negative Tests Were Obtained

... Cleveland, Ohio.—Through the kindness of Dr. John A. Toomey, ... flies were collected in Cleveland and sent to us during the latter ... at a time when poliomyelitis was epidemic in that city. The fly ... close proximity to the point where sewage laden creeks emptied into ... Data with regard to size of population and local cases in this *epidemic* ... difficult to secure and have not been recorded. The samples were ... No virus was recovered.

... *Connecticut (Bridgeport).* During the late summer and fall, ... poliomyelitis were reported in Bridgeport, Connecticut. Two adjacent ... 2 paralytic cases (onset Sept. 1 and 2), and in this epidemic ... 14 ... children there appeared to have been 4 or 5 cases of sus... poliomyelitis with onsets between Aug. 31 and Sept. 2. There were ... this community. A catch of flies was made on Sept. 10, and again on ... a fly trap set in the yard back of one of the houses. The samples were ... No virus was isolated.

... *Pennsylvania.*—Through the kindness of Dr. Joseph ... Dr. Rachel M. Whitlock of Philadelphia, we were notified of an unusual ... which occurred in late August at a children's camp in ... Pennsylvania. The population here consisted of 112 children and 25 ... latter half of August several cases of poliomyelitis occurred, for the ... the last few days in which the camp was open. It was closed on ... 27. It ... clear how many children developed the disease because a number ... returning home. Probably there were 6 or 8 fairly definite cases ... to the camp was made on Sept. 9. Flies collected on Sept. 17 and Oct. ... after the last patient had left were negative for virus.

... This is a boy scout camp in rural Connecticut, recruited ... the eastern part of the state and with an average population of ... of 1 week's duration. Presumably 2 cases of polio... members of this group during the summer, with onsets on ... 2 ... the latter case occurring in a boy who had left the camp 4 days ... trapped in the deserted camp ground on Sept. 15. The ... virus.

... were all obtained from rural or semirural homes in ... In each of these homes one case of poliomyelitis had ... to 20 days prior to the time that the fly samples were ... present. All samples proved negative for the

DISCUSSION

The four positive samples of flies listed in this paper, together with a larger number of similar positive samples reported by Sabin and Ward (3), and the single positive sample reported by Toomey *et al* (4), leave little doubt that the virus of poliomyelitis can be detected not infrequently either in or on the surface of certain flies during epidemic times From our own limited experience it has been easier to find the virus in association with these insects during epidemics than after the epidemic was over

A pertinent question which arises from this finding is Does the virus multiply within the body of certain flies or is it merely carried on their surfaces or or in their alimentary tracts? We do not have sufficient data at our disposal to answer this question but one can point out that in at least three of our four positive samples of flies the insects were collected within a short distance (a few feet to a few yards) from a potential (though not proven) source of human virus in the form of freshly passed human feces. In none of the sites where negative samples of flies were obtained, did we discover an obvious source of fresh human feces which could have been recently passed by a proven carrier, but it is important to point out that the negative sites were not studied with the care that was used with the positive sites. In general nevertheless our findings suggest that mechanical transfer of the virus by flies could have been responsible for these positive tests, and it is unnecessary (for us) to postulate that multiplication of the virus within the fly must have occurred. Actually, however, this second possibility remains to be investigated. The futility of attempting to answer the question by the limited data in this paper is of course apparent, particularly as we still know little of the amounts of poliomyelitis virus in nature,—or their sources. For it is quite possible (in spite of the lack of proof) that there are sources of poliomyelitis virus in nature other than those which come directly from man

We believe therefore that it would be unwise to read any epidemiological implications into these findings, for the presence of the virus in these particular samples of flies could be entirely a *resultant*, and not a *causal* factor in human poliomyelitis Our findings to date therefore, merely indicate that under certain circumstances the virus is carried by flies and we suspect their feeding habits to be responsible. At least, in most of the instances in which the virus was isolated in this series of tests, the flies had had the opportunity of feeding upon fresh human feces which might have contained poliomyelitis virus

CONCLUSIONS

During the summer and fall of 1941 19 samples of flies were collected in epidemic areas both during and after epidemics of poliomyelitis.

Of 8 samples collected for the most part during the latter part of a local

collected [illegible] within 10 days of the onset of a local case of poliomyelitis, 4 yielded the virus, whereas of 8 samples collected more than 10 days from the onset of the last local case, none yielded the virus

In 4 instances there was a potential (though not proven) source of virus (in the form of [illegible] exposed" human feces of recent origin) within a few yards or few feet of the sites where the collections were made Collections of flies from 3 of these sites yielded the virus

No attempt is made in this paper to develop epidemiological implications from this finding

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THE INFLUENCE OF BIOTIN UPON SUSCEPTIBILITY TO MALARIA

By WILLIAM TRAGER, PH.D

(*From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey*)

PLATES 22 AND 23

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That individuals differ in their degree of susceptibility to malaria has long been an accepted fact. Such differences have been ascribed to many and varied factors but it has never been possible to demonstrate a direct relationship between any particular factor and the degree of susceptibility. Nutritional status has received special emphasis in this connection, and it is said that "nutrition is of the greatest importance in the reaction to malaria" (1). There has been, however, but scant clinical or epidemiological evidence, and no experimental evidence, in favor of this idea. While it is true that malnutrition and famine frequently accompany severe epidemics of malaria, it is as likely that the malaria brings about the famine as that the famine makes the malaria more severe. Moreover, as pointed out by Russell (2), famine conditions may indirectly increase the severity of a malaria epidemic by causing malarious individuals to attempt to work before they are fit, increasing the relapse rate, or by driving infected families into other areas, which thus become seeded with gametocyte carriers. The only experimental data on the subject are those of Passmore and Sommerville (3). They found that *Macacus radiatus* monkeys, kept on a diet similar to that of the rice-eating poor of India and especially deficient in vitamins A and C and in calcium, did not develop more severe infections with the monkey malaria parasites *Plasmodium cynomolgi* and *P. knowlesi* than did control monkeys on an adequate diet.

As we have already reported in a preliminary way (4), experiments with avian malaria have now shown that the level in the host animal of biotin (5), an essential growth factor, influences greatly the severity of the infection. Moreover, the level of biotin in the blood of chickens and ducks infected with *Plasmodium lophurae* has been found to increase during the course of the acute infection and to return to normal when the infection subsides.

Methods

Young Rhode Island Red chickens and White Pekin ducks were rendered biotin deficient by feeding them a diet containing a large proportion of dried egg white (6). In the simplest type of experiment, the control animals, which were of the same breed, age, and group as the deficient ones, received the same diet with the egg white replaced by casein. In other experiments the control animals were fed the egg white diet, but they received biotin concentrate by mouth or by intraperitoneal injections. When

the animals were 2 to 4 months old and those on unsupplemented egg white diet showed signs of biotin deficiency, they were all inoculated intravenously with appropriate doses, proportional to their body weight, of malaria parasites

Plasmodium lophurae (7) was used for most of the experiments This parasite, if inoculated intravenously in sufficiently large numbers, produces very heavy infections in ducks (8, 9) and in baby chicks, but only mild infections in older chickens (7, 10–12) The experimental animals were inoculated with an amount of heparinized blood from a heavily infected chicken or duck sufficiently large to permit an accurate parasite count to be made on a thin blood film prepared immediately after inoculation The number of parasites per 10,000 red blood cells was determined in the usual manner (13) on the initial blood films and on blood films prepared daily beginning with the 2nd or 3rd day and continuing through the 5th, 6th, 7th, or 8th day after inoculation

Since relative number of parasites was used as the measure of intensity of infections, it was obviously important to be certain that the blood volume and red blood cells per cubic millimeter at the time of inoculation were approximately the same in experimental and control animals Direct measurements showed that ducks kept on egg white diet for 2 weeks had the same amount of red blood cells per cubic millimeter as those not deficient in biotin Similarly, chicks which were biotin-deficient after more than 4 weeks on egg white diet had a hemoglobin of 85 per cent by the Tallquist method, just as did the control chicks on the casein diet The initial parasite counts themselves, made in every experiment from blood films prepared immediately after inoculation of the animals, provide the most conclusive evidence that the proportion of red blood cells and the blood volume in relation to body weight were entirely comparable in biotin-deficient and non-deficient animals If, for example the biotin-deficient animals had been, at the time of inoculation, anemic as compared to the non-deficient animals, then the injection into them of doses of parasites at the same rate, in proportion to body weight, as for non-deficient animals would have yielded higher initial relative parasite counts for the deficient than for the non-deficient animals Actually, the average initial parasite densities for the different groups of animals in each experiment were remarkably uniform (See, for example, Tables I and II, Charts 1 to 5)

Biotin assays were made on the blood of some of the animals before inoculation and during the course of the infection

Diets—The animals to be made biotin deficient and the control animals were supplied daily with equivalent amounts of food, and usually most of it was consumed by the next day The following diets were used (composition given in percentage by weight)

A A regular baby chick mash Yellow corn meal 25 5, wheat bran 8 5, wheat middlings 8 5, ground wheat 17 1, pinhead oats 17 1, soybean oil meal 2 6, meat scrap 6 0, skim milk 4 3, alfalfa leaf meal 8 5, limestone flour 1 2, fortified cod liver oil 0 3, salt 0 4

B A standard duck food Yellow corn meal 25, wheat bran 25, wheat middlings 25, meat scrap 25 Stock ducks received this diet as a moist mash mixed with a little chopped lettuce When this diet was used in experiments, it was fed dry without any lettuce

1 Yellow corn meal 40, wheat middlings 25, bran 10 powdered egg white 25, plus a small amount of grits and oyster shell.

1*a* Same as 1, but egg white replaced by washed casein.

2 (After (6)) Yellow corn meal 48 wheat middlings 24 powdered egg white 24, bone meal 1.5, cod liver oil 1, salt 0 5 ground oyster shell 1

2*a* Same as 2, but egg white replaced by washed casein.

3 Diet B 75, powdered egg white 25

3*a*. Same as 3, but egg white replaced by washed casein.

3*b* Same as 3*a* but casein first mixed with riboflavin to provide 5 mg. riboflavin per 100 gm casein

4 Diet A 75, powdered egg white 25

4*a*. Same as 4 but egg white replaced by washed casein.

4*b* Same as 4*a*, but casein first mixed with riboflavin to provide 5 mg riboflavin per 100 gm casein

4-I Diet 4 mixed with enough biotin concentrate (S.M.A Co No 1000) to supply 500 γ per kg of food in addition to the biotin of the food This left little excess biotin over that which combined with the egg white.

4-II Diet 4 mixed with enough biotin concentrate to supply 1 mg biotin per kg of food in addition to the biotin in diet 4 This left an excess, over that which com bined with the egg white of about 40 γ per 100 gm. of diet.

5 (After (14)) Yellow corn meal 58 parts, wheat middlings 25 parts, washed casein 12 parts. These three ingredients were mixed and heated in shallow layers in an oven at 120°C for 30 hours. They were then mixed with one part each of $CaCO_3$ $CaHPO_4$ $2H_2O$ and NaCl and with the following amounts of vitamins per kg of ration thiamin 1 mg riboflavin 1 mg., 2 methyl-1 4-naphthoquinone 10 mg

5-I Diet 5 supplemented with 15 mg calcium pantothenate per kg (15)

Animals on diets 3 3*a* 3*b*, 4 4*a* 4*b* 4-I, 4-II 5, and 5-I all received once a week 3 to 4 drops of haliver oil with viosterol.

Strains of Parasites—The strains of *P lophurae* were all derived from strain 12A of Coggeshall (16) One strain has been maintained in baby chicks and passed by intracerebral inoculation every 6 days It represents the original 12A strain except that it was passed once through *Aedes aegypti* mosquitoes before being returned to chicks and used for the present experiments. It was used for all the tests with chickens. Three sub-strains have been maintained in ducks. One (D 1) was de rived from the original chick strain before mosquito passage and was used only for Experiment 1 of the experiments described in this paper The second strain (D 2) was obtained from D-1 by infecting *Aedes albopictus* mosquitoes and then allowing them to feed on a duck This strain used in Experiments 2 and 11, has consistently been less virulent than D-1 and than most of the duck strains described in the litera ture The third strain (D 3) was derived from the chick strain after mosquito passage and is about as virulent as strain D-1 It has been used for the biotin injection experiments. All the duck strains were passed by intravenous inoculation every 5 to 6 days.

Strain 3T of *Plasmodium cathemerium* in ducks was obtained through the kindness of Dr Fruma Wolfson.

Inoculation of the Animals—In each experiment the same infected blood was used

for the inoculation of all the animals Chickens were infected with *P lophurae* by injecting them in the neck vein with pooled heparinized blood from 1 week old chicks which had been infected 5 days previously Ducks were infected *via* the neck or leg vein with heparinized blood, taken on the 5th day of infection, from a single donor duck 2 to 8 weeks old

Biotin Assays—The microbiological assay method of Shull, Hutchings, and Peterson (17) was used, with some minor modifications The preparation of the basal medium was modified in such a way as to permit its use for the assay of pantothenic acid as well as biotin The yeast extract was prepared by dissolving 20 gm of Difco bacto-yeast extract in 200 ml of 0 5 N sodium hydroxide and autoclaving for 30 minutes at 15 lbs, as in the pantothenic acid assay method (18) The solution was neutralized with glacial acetic acid, boiled, and filtered The filtrate was diluted to 1 liter, brought to pH 2 with concentrated sulfuric acid, and treated with norit A and then with Superoxol in the manner described by Shull *et al* (17) The adenine guanine-cystine solution was prepared using the amounts given by Landy and Dicken (19) The stock solution mixture for biotin assay was as follows H_2O_2-treated hydrolyzed casein solution, 200 ml, alkali and H_2O_2-treated norit yeast filtrate, 200 ml, vitamin solution (as described in (17) but exclusive of calcium pantothenate), 4 ml, calcium pantothenate solution (20 mg dissolved in 50 ml water), 1 ml, adenine guanine cystine solution, 200 ml, tryptophane, 150 mg plus asparagine, 400 mg, first dissolved in 50 ml hot water, and water to make a total volume of 1 liter For pantothenic acid assay the stock solution mixture was prepared in the same way except that the calcium pantothenate was omitted and biotin was added as 0 4 ml of a solution of 25 γ of the free acid per ml

Total biotin (20) was always determined The sample of 0 5 ml of blood, plasma, or red cells, or 0 5 gm of minced liver, was autoclaved for 1 hour at 15 lbs in 5 ml of 3 N sulfuric acid The material was filtered, the residue washed with a little distilled water, and the combined filtrates neutralized with 10 N sodium hydroxide and diluted to 15 ml with distilled water Such preparations from blood were assayed at concentrations of 0 4, 0 7, and 1 0 ml per 10 ml of culture medium Preparations from liver had to be greatly diluted with water and were likewise assayed at three different levels Material for pantothenate assay was autoclaved at neutrality in water and was tested at three different dilutions

Lactobacillus casei ε (American Type Culture Collection No 7469) was used throughout It was maintained in culture following the method of Snell and Strong (21) and the inoculum was prepared after the manner of Landy and Dicken (19) For each series of biotin assays, a standard curve was prepared from the results with tubes containing known amounts of biotin ranging from 0 05 to 1 0 mγ per 10 ml Growth was determined by titrating with 0 1 N sodium hydroxide the acid produced in the cultures after 3 days' incubation at 38°C

In a recovery experiment, 2 5 mγ of pure biotin were added to each of six 0 5 ml samples of chicken blood These were then assayed for biotin together with corresponding 0 5 ml samples of blood, to which no biotin had been added, from the same six chickens The greatest error in recovered biotin was 25 per cent, and the average for the 6 determinations was 2 57 mγ biotin recovered

The Effect of Egg White Diets on Susceptibility of Ducks and Chickens to P lophurae

Charts 1 to 5 give the results of four typical experiments which illustrate the effect of biotin deficiency, induced by a high egg white diet, on the susceptibility of ducks and chickens to *P lophurae* It is apparent that, for both species of

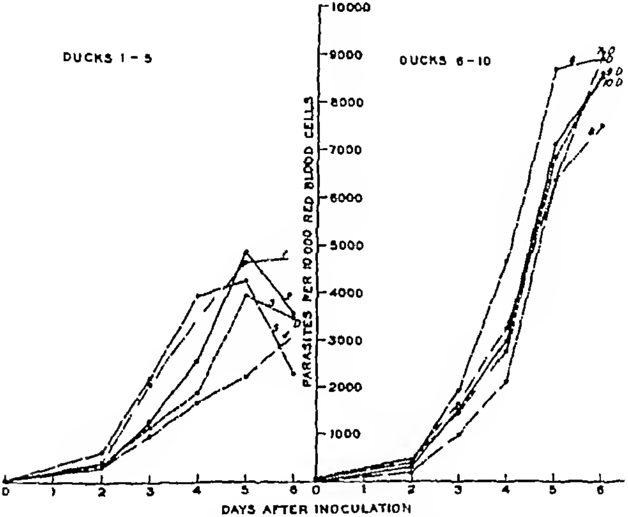

CHART 1 Experiment 1 Ducks 1 to 5 fed casein diet 1a, ducks 6 to 10 egg white diet 1 Inoculated with *P lophurae* when 14 days old Average weights when inoculated 1 to 5 105 gm , 6 to 10, 120 gm In this and succeeding charts, D signifies that animal died on day of last parasite count.

hosts, the average peak parasite number was 50 to 100 per cent higher in the biotin-deficient animals than in the controls, the highest peak was always reached in a deficient animal and the lowest in a control, and more of the deficient animals died of the infection Appropriate uninfected control animals very rarely died of biotin deficiency alone when only 3 to 4 weeks old It is noteworthy that in Experiments 1 to 4 the biotin-deficient and the control animals differed but little in weight Indeed, in Experiments 1 to 3 the animals on egg white diet weighed a little more than the controls It seemed probable

that the egg white supplied an unusually large amount of riboflavin, and accordingly in other experiments the control casein diets (as 3*b*, 4*b*) were supplemented with riboflavin at the rate of 5 mg per 100 gm of casein When this was done, the animals on casein diet always grew more rapidly than those on egg white diet, but the responses to infection with *P lophurae* were exactly the same as when additional riboflavin was not supplied

When the young chickens had been on an egg white diet for about 2 weeks they began to show the syndrome first described by Ringrose, Norris, and

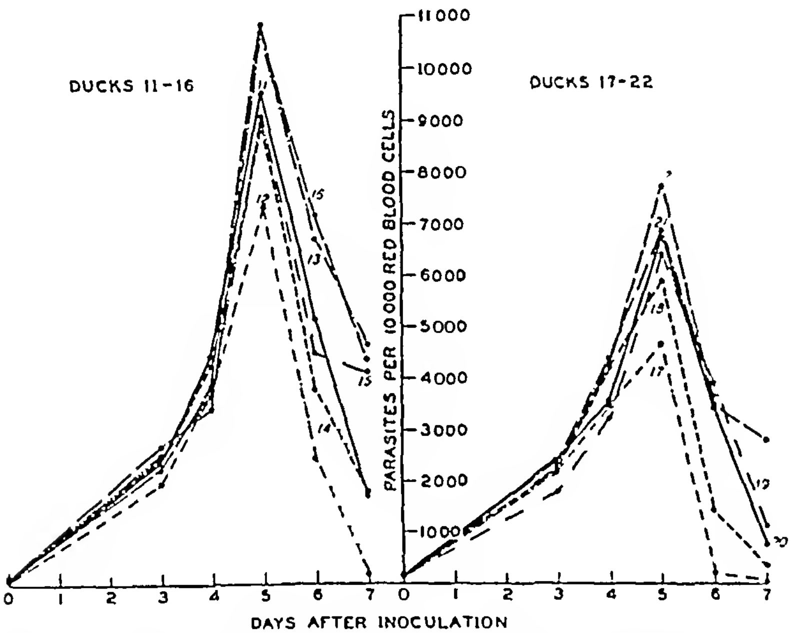

CHART 2 Experiment 2 All fed diet B for first 4 days Thereafter ducks 11 to 16 were fed egg white diet 3, while ducks 17 to 22 were continued on diet B Inoculated with *P lophurae* when 13 days old Average weights when inoculated 11 to 16, 127 gm , 17 to 22, 98 gm

Heuser (22) and illustrated in Fig 1 The down and feathers of the deficient chicks had a generally rough appearance, the feet showed a marked scaly dermatitis, and lesions appeared at the corners of the mouth and over the eyes Some individuals developed perosis and in some the upper portion of the beak grew in a curved manner resulting in malocclusion The animals on the various casein diets showed no signs of dermatitis The early lesions of biotin deficiency in ducks are illustrated in Fig 2 The down on the face, neck, and back of ducks on egg white diet presented a matted, rough appearance and sometimes fell, or was pulled, out, leaving bald areas Lesions appeared around the eyes of the animals and their legs were frequently bowed They were, however, just as

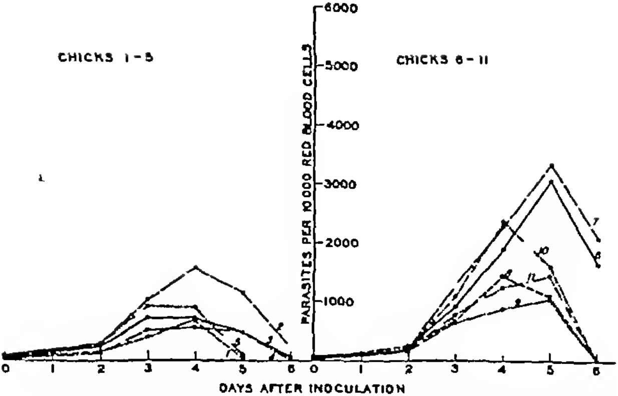

CHART 3 Experiment 3 All fed diet A for first 5 days Thereafter chicks 1 to 5 were fed casein diet 2a and chicks 6 to 11, egg white diet 2 Inoculated with *P lophurae* when 15 days old Average weights when inoculated 1 to 5 92 gm. 6 to 11, 98 gm

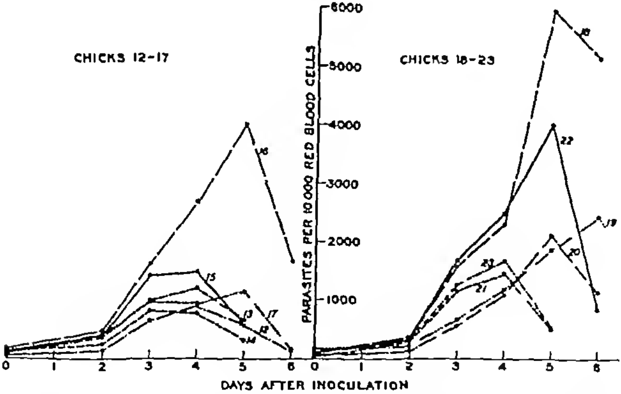

CHART 4 Experiment 4 Chicks 12 to 17 fed casein diet 2a chicks 18 to 23 fed egg white diet 2 Inoculated with *P lophurae* when 22 days old. Average weights when inoculated 12 to 17, 133 gm. 18 to 23 105 gm.

active, when 3 to 4 weeks old, as the control animals Ducks kept on a casein diet, such as 3*b* or 4*b*, eventually developed signs similar to those of ducks on egg white diet but much less marked Rough down and bowing of the legs occasionally appeared even in ducks kept on diet B supplemented with lettuce, but all these abnormalities appeared regularly, early, and to a marked degree, only in ducks fed a high egg white diet

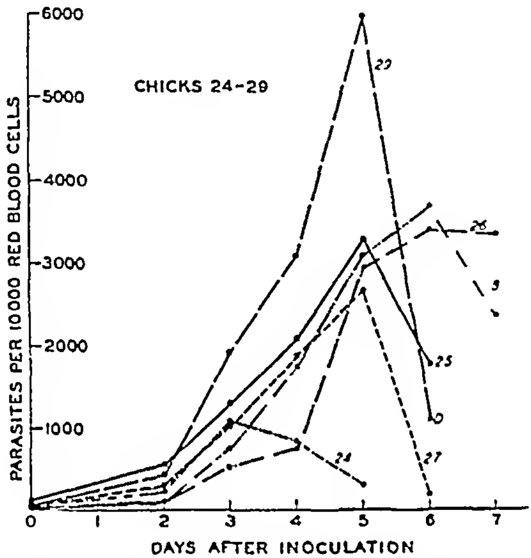

CHART 5 Experiment 4 Chicks 24 to 29 fed casein diet 2*a* for first 5 days, thereafter egg white diet 2 Inoculated with *P lophurae* when 22 days old Average weight when inoculated, 122 gm For controls see Chart 4, chicks 12 to 17

The Effect of Pantothenic Acid Deficiency and of Small Degrees of Biotin Deficiency

Since the biotin-deficient animals eventually die of the deficiency, it seemed possible that their greater susceptibility to *P lophurae* might be merely the result of some general lowering of resistance, not necessarily specifically associated with the biotin level It was therefore of interest to weaken the animals by means of some other nutritional deficiency Pantothenic acid was selected since it is essential for the growth of chicks and since a deficiency of it produces lesions at the corners of the mouth and on the eyelids somewhat resembling those seen in biotin deficiency Chickens maintained on the heated diet 5 showed the typical signs of pantothenate deficiency (Fig 3) and were smaller and weaker than comparable chicks fed on egg white diet The control chicks, which received diet 5-I, were vigorous and normal in every respect except for a slight scaliness on the feet Ducks fed the pantothenic acid-de-

ficient diet (Fig 4) grew very poorly and soon became weak and unable to open their eyes Several died when they were only 2 weeks old and before they had been inoculated with malaria parasites The control ducks fed diet 5 I appeared entirely normal The average pantothenate content of the blood was, in mγ per ml., 167 for 2 deficient ducks and 293 for 3 ducks on the adequate diet Yet in spite of the great difference in general health between the animals deficient in pantothenic acid and those not deficient in this vitamin, the former did not develop any more severe infections with *P lophurae* than did the latter The result obtained with the chicks (Chart 6) was especially instructive. It is at once apparent that, while the small, weak, pantothenic acid-deficient

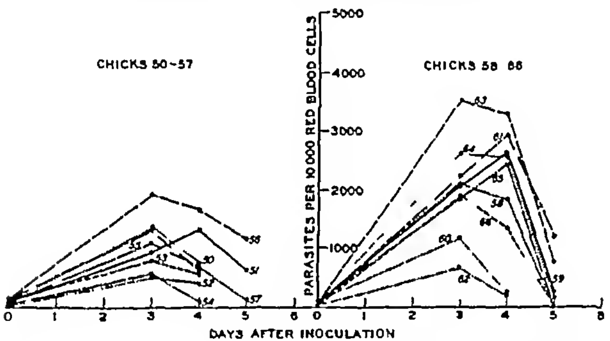

CHART 6 Experiment 5 Chicks 50 to 57 fed heated diet 5, deficient mainly in pantothenic acid chicks 58 to 66 fed diet 5-I (diet 5 + 15 mg calcium pantothenate per kg) Inoculated with *P lophurae* when 25 days old Average weights when inoculated 50 to 57, 96 gm. 58 to 66, 133 gm

chickens had mild infections, exactly of the type to be expected in chickens of their age, 5 out of 9 large, vigorous animals on the diet supplemented with pantothenate developed unexpectedly heavy infections and only 2 had very mild infections As has already been stated, the chickens not deficient in pantothenic acid showed a scaly dermatitis of the feet which is a characteristic sign of partial biotin deficiency It has recently been shown (23) that a heated diet supplemented with thiamin riboflavin, 2 methyl-1 4-naphthoquinone, and *calcium pantothenate, such as diet 5-I is still somewhat deficient in biotin* It has also been frequently observed (24 23) that rapidly growing animals show the most distinct signs of biotin deficiency It therefore seems likely that the pantothenic acid-deficient chicks, which failed to grow well, received enough biotin from the diet, while the chicks which received adequate pantothenic acid

and which grew rapidly were unable to obtain enough biotin Their partial biotin deficiency would then account for their greater susceptibility to infection with *P lophurae* —

Other types of experiments provide still better evidence for the effects of small degrees of biotin deficiency on the severity of *P lophurae* infections

In Experiment 6 (Chart 7) one group of chickens was kept on casein diet, one on egg white diet, and one on egg white diet supplemented with biotin (diet 4-I) The amount of biotin added in diet 4-I was calculated on the power of commercial egg white to inactivate biotin (25) and the average biotin content of chick feeds, and should have been enough to leave 20 γ per 100 gm of diet For about the first 10

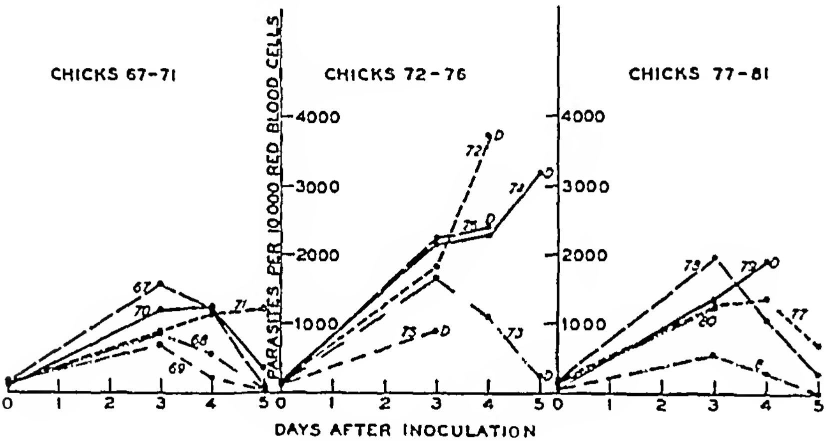

CHART 7 Experiment 6 Chicks 67 to 71 fed casein diet 4*a* for first 16 days, thereafter casein diet 4*b*, chicks 72 to 76 fed egg white diet 4, chicks 77 to 81 fed egg white + biotin concentrate diet 4-I for first 18 days, thereafter egg white + biotin concentrate diet 4-II Inoculated with *P lophurae* when 25 days old Average weights when inoculated 67 to 71, 176 gm, 72 to 76, 122 gm, 77 to 81, 149 gm

days, the chicks fed diet 4-I showed even better growth than those fed the casein diet 4*a* But by the time they were 2 weeks old they began to show signs of biotin deficiency which were almost as bad as those shown by the chicks on plain egg white diet 4 It was apparent that the biotin which had been added was inadequate Woolley and Longsworth (26) had in the meantime reported almost twice as high a biotin-inactivating power for pure antibiotin (avidin) as had been reported by Eakin, Snell, and Williams (27) The amount of biotin concentrate added to diet 4 was therefore doubled when the chicks were 18 days old Although they still showed signs of biotin deficiency when they were inoculated a week later with *P lophurae*, they developed less severe infections than the chicks on unsupplemented egg white diet and slightly more severe infections than the chicks on casein diet

In Experiment 7 (Table I) all 21 chicks were maintained from the day of hatching on a high egg white diet (diet 4) One group of 7 chicks received no other treatment

These chicks all showed definite signs of biotin deficiency by the time they were inoculated with *P lophurae* The 7 chicks of the 2nd group received daily by intra peritoneal injection enough biotin concentrate (S M.A Co No 1000) diluted with 0 85 per cent salt solution to furnish 1 γ of biotin per chick. These chicks grew well and showed no signs of biotin deficiency other than a distinct but not at all severe, scaly dermatitis of the feet. Each chick of the 3rd group received intraperitoneally enough biotin concentrate to furnish 3 γ daily for the first 17 days and 6 γ daily thereafter Five of these 7 chickens showed no signs of biotin deficiency at the time they were inoculated in agreement with the reported biotin requirement of chicks of about 3 to under 10 γ (28 29) The other 2 had been exceptionally small, weak chicks since the date of hatching and they failed completely to respond to the biotin injections When they were 20 days old they were as small and showed as distinct

TABLE I

Experiment 7 The Effect of Intraperitoneal Injections of Biotin Concentrate on the Course of P lophurae Infections in Chickens Fed Egg White Diet 4 and Infected When 20 Days Old

7 chickens in each group

Treatment	Average weight when inoculated	Parasites per 10,000 red cells				No of chickens	
		Initial No		Peak No		Parasites cleared out by 5th day	*Died*
		Range	Average	Range	Average		
	gm						
No biotin	114	120–250	177	3750–12 120	7400	0	3
1 γ biotin daily	131	135–215	181	3860–7480	5600	0	0
3 γ biotin daily for 17 days, then 6 γ biotin daily	150	160–295	214	2380–7920 (2380–5500)*	4900 (3900)*	2	0

* These values obtained if results with 2 obviously deficient chicks are omitted from this group.

signs of biotin deficiency as did the chicks in the group receiving no biotin. All the chicks were inoculated with a very large dose of *P lophurae* By far the highest peak parasite number occurred in the group receiving no biotin and the lowest in the group receiving the larger amount of biotin. The average peak parasite number was highest for the no biotin group lowest for the high biotin group, and intermediate for the low biotin group Especially interesting was the fact that the 2 chicks in the high biotin group which failed to respond to the biotin injections both had peak parasite numbers over 7,000 If the results with these 2 chicks are omitted the range and average peak parasite numbers for the group become considerably lower Only in the group receiving high biotin were there any chicks which had very few parasites by the 5th day (5 and 20 per 10 000 red cells) In the other two groups, and especially in the group receiving no biotin the parasite number was still up in the thousands on the 5th day Deaths from the infection occurred only in the group receiving no biotin, where 3 out of the 7 chickens died 5 6 and 8 days respectively after inoculation

In this experiment the chickens receiving only 1 γ of biotin daily were active, well grown, and of normal appearance except for the mild scaliness of the feet Yet they had more severe infections than the chickens receiving more nearly adequate amounts of biotin It is evident that in the presence of a small degree of biotin deficiency the administration of biotin may be considered as a specific measure lessening the severity of the infection with *P lophurae*

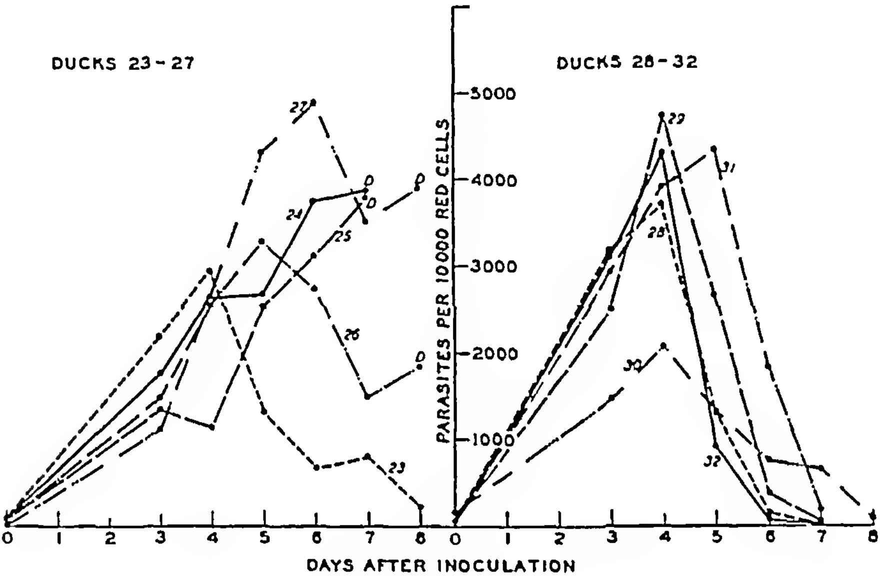

CHART 8 Experiment 8 Ducks 23 to 27 fed egg white diet 4, ducks 28 to 32 fed casein diet 4*b* Inoculated with *P cathemerium* strain 3T when 21 days old Average weights when inoculated 23 to 27, 200 gm , 28 to 32, 349 gm Average initial parasites per 10,000 red cells 23 to 27, 79, 28 to 32, 91

The Influence of Egg White Diets on Susceptibility to P cathemerium

The effect of biotin deficiency on susceptibility has been studied with two species of bird malaria parasites other than *P lophurae* Experiments with *P cathemerium* strain 3T gave especially interesting results, illustrated in Chart 8

In the non-deficient ducks on the casein diet, the parasite count rose very rapidly for the first 4 days and then fell off equally abruptly As is usual with *P cathemerium* infections in ducks, none of the animals died (30) The infections in the biotin-deficient ducks did not attain any higher peaks than in the non-deficient ones In deed, the parasite count at first rose more slowly in the former than in the latter animals But in only one (No 23) of the deficient animals was the parasite peak

reached on the 4th day and followed by an abrupt decline in the number of parasites. In one duck (No 26) the parasites increased to a peak on the 5th day, fell off some what on the 6th and 7th days and rose again on the 8th day, when the animal died. In one (No 27) the parasites increased to a high peak on the 6th day, fell off slightly on the 7th day, and rose again on the 8th day when the animal died In the other 2 ducks (Nos. 24 and 25) the parasites continued to increase until the 7th day, when both animals died Control uninfected biotin-deficient animals survived 1 to 2 weeks or longer beyond the end of the experiment.

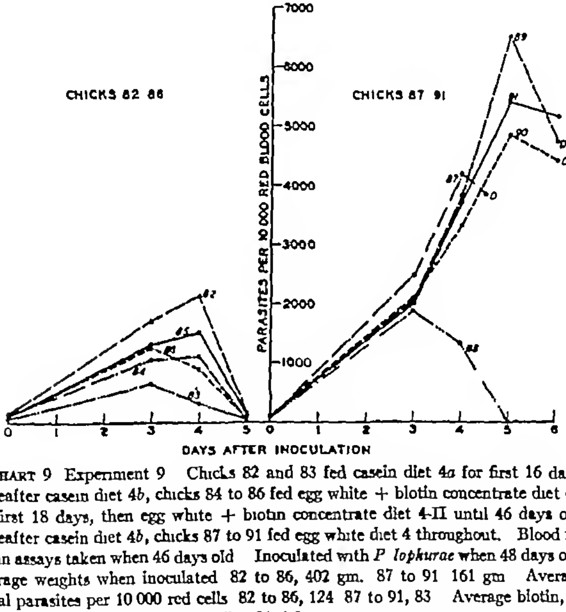

CHART 9 Experiment 9 Chicks 82 and 83 fed casein diet 4a for first 16 days, thereafter casein diet 4b, chicks 84 to 86 fed egg white + biotin concentrate diet 4-I for first 18 days, then egg white + biotin concentrate diet 4-II until 46 days old thereafter casein diet 4b, chicks 87 to 91 fed egg white diet 4 throughout. Blood for biotin assays taken when 46 days old Inoculated with *P lophurae* when 48 days old. Average weights when inoculated 82 to 86, 402 gm. 87 to 91 161 gm Average initial parasites per 10 000 red cells 82 to 86, 124 87 to 91, 83 Average biotin, as mγ per ml of blood 82 to 86 3 0, 87 to 91 1 2

Thus, in the non-deficient animals the parasites increased very rapidly at first, but were then equally rapidly removed from the circulation, while in the biotin-deficient ducks the parasites at first increased more slowly, but they continued to increase and could not be successfully cleared out of the blood stream

The Biotin Content of the Blood in Relation to Infections with P lophurae

The question arises as to what precisely are the biotin levels associated with different degrees of susceptibility A small beginning toward answering this question has been made with the parasite *P lophurae* Although it was recognized that the level of biotin in the blood probably would not give an entirely accurate indication of the biotin level of the body as a whole, most of the biotin determinations were made on blood, since samples of this could be readily obtained without injuring the animal and since blood is the medium in which the malaria parasites live Chart 9 illustrates the result of an experiment with chickens 48 days old when inoculated

TABLE II

Experiment 10 The Relation between the Average Biotin Content of the Blood before Inoculation and the Average Peak Parasite Number for 3 Groups of 5 Ducks Each on 3 Different Diets, Inoculated with P lophurae When 14 Days Old

Diet	Biotin mγ/ml blood	Average weight when inoculated	Parasites per 10 000 red blood cells		Remarks
			Initial	Peak	
		gm			
Egg white diet 4	1 8	167	116	8360	2 ducks still had high parasite counts (2940 and 6680) 11 days after inoculation and they died on the 13th day
Casein diet 4a for first 11 days, then 4b	3 0	118	115	6544	In both of these groups, the highest parasite count on the 11th day after inoculation was 115 Most of the ducks showed no parasites at this time and none died
Casein diet 3a for first 11 days, then 3b	3 0	149	110	6680	

Four of the 5 chickens in the group averaging before inoculation only 1 2 mγ of biotin per ml of blood developed infections three times as severe as those developed by the 5 chickens with an average biotin of 3 0 mγ per ml of blood Three of the chickens with the low biotin died before the 7th day after inoculation and a fourth (No 91) was killed on the 8th day, when it was very weak

Table II illustrates again, for ducks, the inverse relation between blood biotin level and the average peak parasite number Note the constancy of the results obtained with the two groups of ducks on two very different kinds of casein diets, which however had in common the property of not interfering with the biotin supply of the animals

Since *P lophurae* produces heavier infections, after the inoculation of comparable large doses, in ducks and in baby chicks than in older chickens, it was of

interest to compare the biotin levels in these 3 groups of animals The biotin level of the blood of chickens kept on adequate diets did increase after they were 1 month old (Table III) However, the increase in the resistance of chickens to *P lophurae* infections is quite notable by the age of 2 weeks, and at this time there appeared to be no higher biotin level either in the blood or in the

TABLE III

Changes with Age in the Biotin Content of the Blood of Individual Chickens on Egg White and Control Diets

Diet	Chick No	Biotin mγ/ml. blood at	
		31 days	46 days
Casein diet 4a for 14 days, then 4b	82	2 7	5 1
	83	2 8	4 6
Egg white diet 4 + 500 γ biotin per kg for 16 days, then 1000 γ per kg	84	1 2	2 3
	85	1 6	2 4
Egg white diet 4	87	1 8	1 2
	88	1 0	1 3

TABLE IV

The Biotin Content of the Blood and Liver of Young Ducks and Chickens

Age in days	Biotin per ml blood		Biotin per gm. liver	
	Individual values	Average	Individual values	Average
	mγ	mγ	mγ	mγ
Ducks				
4	4 3 4 0 4 1	4 1	1260, 1540 1720	1507
25	3 0 3 2, 3 4	3 2	854 1020 1450	1108
67	2 3	2 3	1950 1630	1790
88	4 9	4 9	2960	2960
Chicks				
2	2 6 2 2 2 2	2 3	2516, 2580 3274	2790
14	3 0 2 4 3 2	2 9	2520 1778 1860	2053
26	2 5 2 5 2 3	2 4	2854 1688 3374	2639

liver than in 2 day old chicks (Table IV) Also as shown in Table IV, the blood level of biotin is actually a little higher in ducks than in young chickens The concentration of biotin in the liver of young ducks was found to be little more than half of that in the liver of young chicks, and it may well be that the general body level of biotin is higher in the latter than in the former

In any case, the idea that a single static concentration of biotin in the blood determines the degree of susceptibility to *P lophurae* infection is much too

simple to be expected to work Rather one might expect that the important factors are the extent and nature of the reserve supply of biotin in the body, and the speed with which biotin can be mobilized into the blood stream Some evidence has been obtained which shows that infection with *P lophurae* modifies temporarily the biotin level of the blood of both chickens and ducks

This was first observed in assays made with the chickens of Experiment 6 (see Chart 7) Besides the 5 chickens on each of the 3 diets which had been infected when 25 days old, several other chickens in each group were left uninfected (used later for Experiment 9) When the infected chickens were in the 6th day of their infection (except for chicks 73 and 80 which were bled on the 5th and 3rd day respectively),

TABLE V

The Biotin Content of the Blood of Uninfected 31 Day Old Chickens Kept on Egg White and Control Diets, and of Similar Chickens just Recovered from Infection with P lophurae

Diet	Condition	Hemoglobin	Chick Nos *	Biotin per ml blood	
				Range	Average
		per cent		$m\gamma$	$m\gamma$
Casein diet 4*a* for 14 days, then 4*b*	Not infected	85	82, 83	2 7–2 8	2 8
	Infected	55	67 to 71	4 0–6 9	4 9
Egg white diet 4	Not infected	85	87 to 91	1 0–2 0	1 6
	Infected	—	73		3 1
Egg white diet 4 + 500 γ biotin per kg for 16 days, then 1000 γ per kg	Not infected	85	84 to 86	1 2–2 0	1 7
	Infected	55	77, 78, 80, 81	3 1–4 4	3 6

* See also Experiments 6 and 9, Charts 7 and 9

and both uninfected and infected ones were 31 days old, 1 ml of blood was taken from each and assayed for biotin, with the results shown in Table V

It is evident that, regardless of diet, animals just recovering from infection with *P lophurae* and suffering from a considerable degree of anemia had about twice as much biotin per milliliter of blood as had uninfected animals A similar result was obtained in assays on blood taken from ducks 6 days after inoculation with *P lophurae* Chart 10 shows the results of a more detailed analysis of this phenomenon carried out with 4 ducks on an adequate diet (diet B with lettuce)

A sample of blood was taken from each duck just before inoculation with *P lophurae* and again on the 4th, 6th, and 8th days after inoculation The blood was assayed for biotin Blood films were made on the 4th, 5th, and 6th days so as to include the day of the peak parasite number Both the plasma and red cell levels of biotin had

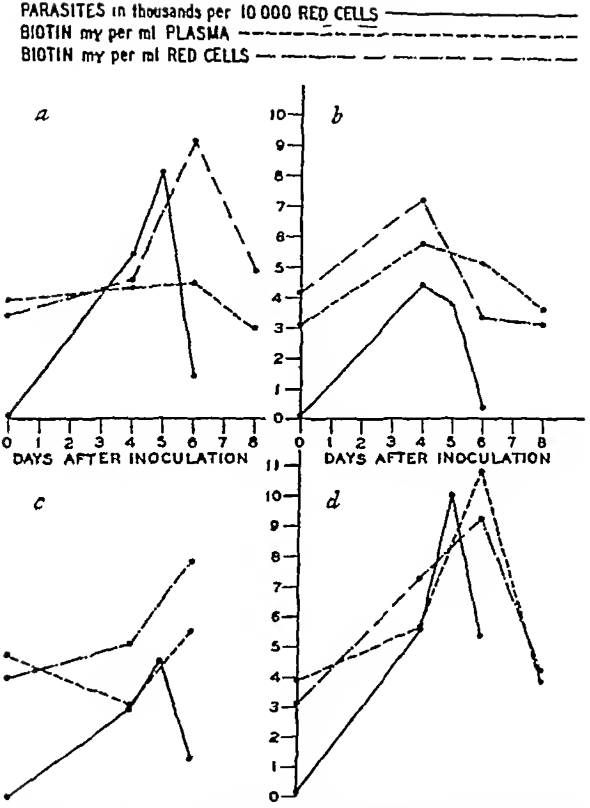

CHART 10 Experiment 11 Changes, during course of infection with *P lophurae*, in the biotin content of the plasma and red blood cells of 4 ducks fed an adequate diet and inoculated when 16 days old.

already risen by the 4th day in all the ducks except duck *c*, whose plasma biotin fell while the red cell biotin rose. In duck *b* whose infection reached its peak on the 4th day, the biotin in both plasma and red cells likewise reached a peak on the 4th day,

fell on the 6th day, and was back to its starting point on the 8th day, when very few parasites remained in the blood In the other 3 ducks the peak parasite number occurred on the 5th day and the highest biotin levels on the 6th day The biotin was again back to its original level by the 8th day (duck *c* died of the bleeding on the 6th day) Moreover, the extent of the increase in biotin level of both red blood cells and plasma was in general proportional to the extent of the parasitemia This rise in biotin level cannot be explained solely on the basis of the new red cells formed in response to the anemia produced by the parasites (assuming that young red cells have a higher biotin content than mature ones) The increased biotin level was already apparent by the 4th day, when there was as yet no large proportion of young red cells, the increase appeared in the plasma as well as in the red cells, and both plasma and red cells were back to a normal biotin level by the 8th day after inoculation, when a large proportion of young red cells was still present

These results suggest that biotin is mobilized into the blood stream during infection with *P lophurae*, and that it may play a rôle in reducing the number of parasites

A single, somewhat similar experiment (Experiment 12, Chart 11) has been performed with 4 chickens, 4 weeks old and weighing about 300 gm at the start of the experiment

Each of 2 of the chickens (*a*, *b*) received by stomach tube on 2 successive days a dose of 0 25 ml of a solution of 0 2 gm of phenylhydrazine hydrochloride in 10 ml of water At the same time that these chickens received their first dose of phenylhydrazine, the other 2 chickens were inoculated intravenously with *P lophurae* Blood for biotin assay was taken into a measured amount of heparin solution, by heart puncture, just before the first phenylhydrazine treatment or inoculation with parasites, and again 1½, 3, and 5 days later This blood was centrifuged in a uniform way, the volume of red cells and plasma was noted, and measured amounts of plasma and red cells were assayed for biotin Stained blood films were prepared daily and the relative numbers of parasites and young red cells were determined The results are summarized in Chart 11

It is apparent that the plasma biotin rose in chickens *c* and *d* just as it did in the ducks of Experiment 11 Especially noteworthy is the fact that the plasma biotin in both chickens had doubled only 1½ days after inoculation Thus, in chickens the biotin in the plasma not only attained higher levels than in the ducks in Experiment 11, but it did so much more quickly If additional results of this type can be obtained, they will support the idea that older chickens infected with *P lophurae* can clear out their infections more rapidly than ducks, partly because they can raise their plasma biotin more quickly and to a greater extent The data for chickens *c* and *d* also show that the changes in plasma biotin are not connected with the anemia which follows the malarial infection The level of biotin in the plasma was much increased 1½ days after inoculation, when there was no anemia, as evidenced by the normal volume per cent of red

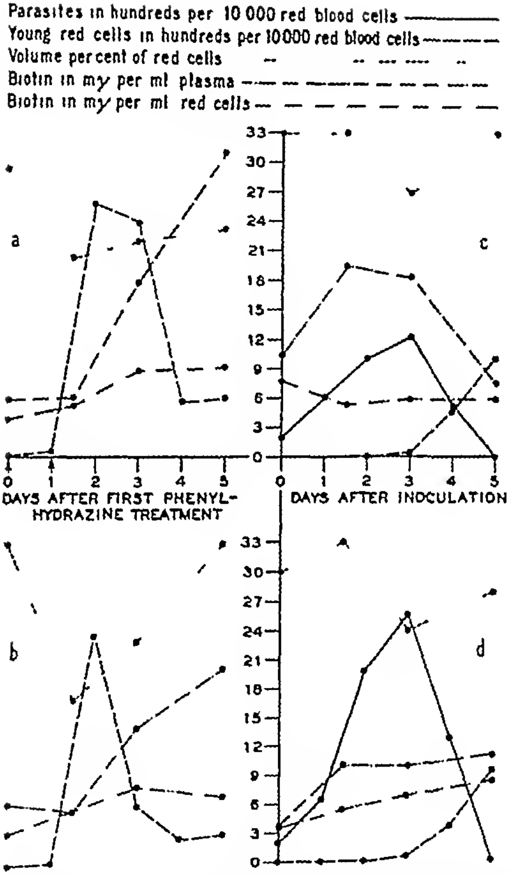

CHART 11 Experiment 12 Changes during infection with *P lophurae* and following phenylhydrazine treatment in the erythrocytes and in the biotin content of erythrocytes and plasma of 4 week old chickens. Chickens *a* and *b* received phenylhydrazine on the days indicated by the arrows, while chickens *c* and *d* were inoculated intravenously with a large dose of *P lophurae*.

cells (33 per cent) and the lack of young red cells In chickens *a* and *b* young red cells were beginning to appear within 1 day after the first phenylhydrazine treatment and there was a marked anemia ½ day still later The level of biotin in the plasma showed no increase at the time, but it was greatly increased on the 3rd day after the first phenylhydrazine treatment and continued to increase to the 5th day Although the increase followed the production of large numbers of young red cells, it continued after the young red cell production had ceased The young red cells themselves did not appear to have a sufficiently higher biotin content than the older ones (as evidenced by the relatively small increase in the biotin level of the red cells) to account for the great changes in the biotin level of the plasma It also does not appear likely that the changes in plasma biotin can be explained directly on the basis of red cell destruction, since the red cells originally contain no higher concentration of biotin than the plasma These results with phenylhydrazine suggest a method for artificially changing the plasma biotin level It is very noteworthy that chickens treated with phenylhydrazine have been observed to develop less severe infections with *P lophurae* than untreated chickens (31) This fact has been ascribed to the apparent preference of *P lophurae* for mature erythrocytes, but the effect of phenylhydrazine in increasing the plasma biotin level may also play a part Canaries treated with phenylhydrazine have been found to develop more severe infections with *P cathemerium* than untreated birds, and this again has been ascribed to the preference of this parasite for young erythrocytes (32, 33) It has already been shown that the initial rate of multiplication of *P cathemerium* is higher in ducks with a normal biotin level than in biotin-deficient ducks, a fact which fits very well with the idea that the effect of phenylhydrazine is partly a result of its action on the biotin level Indeed, since the indications are that immature red cells do have a somewhat higher biotin content than mature ones, the so called preference of different species of malaria parasites for young or old red cells might be intimately connected with the relative biotin content of these cells

Experiments on the Injection of Biotin into Ducks Kept on an Adequate Diet and Infected with P lophurae

A number of preliminary experiments, four with S M A biotin concentrate No 1000 and one with pure biotin (very generously supplied by Dr du Vigneaud) have failed to give consistent results

In 3 of the experiments there seemed to be a small but significant decrease in the severity of the infections in the ducks injected with biotin as compared with the untreated ducks In the other 2 experiments there was no effect of the biotin treatment In one of these experiments biotin assays were made on the blood of treated and untreated ducks, 1 day after inoculation with *P lophurae* and 12 hours after

the last intravenous injection of biotin into the treated animals (S.M.A. biotin concentrate No. 1000 to supply 25 γ of biotin per 100 gm. body weight).

In these assays there was actually more biotin in the blood of the untreated than in the blood of the treated ducks. West and Woglom (34) similarly found that excess biotin injected into mice was very rapidly excreted. Before a conclusive test can be made of the possible effects of a higher than normal blood level of biotin on infection with *P. lophurae* it will be necessary to find a way of artificially maintaining over a period of several days a continuously high level of biotin in the blood.

DISCUSSION

Biotin is evidently a substance which affects the degree of natural susceptibility to malarial infection. How the biotin level of the blood exerts its influence upon the extent of multiplication of the parasite can at present only be guessed. A very simple theory would assume that all species of malaria parasites require biotin for growth (since highly specialized parasites probably cannot themselves synthesize biotin), that for each species of bird malaria parasite there is an optimum range of biotin concentration in the blood of the host, that growth of the parasites is slowed up if the biotin concentration falls below this range, and that growth is inhibited by a direct toxic effect of biotin concentrations above the range. On the basis of such a theory, *Plasmodium lophurae* would require a relatively low optimal concentration of biotin. As the infection progresses it causes the biotin of the blood to increase. When the biotin level exceeds the favorable concentration range, the multiplication of the parasite is greatly reduced and the acute infection is terminated. Biotin-deficient animals are less capable of increasing sufficiently their blood biotin level, and so more of them cannot bring their infections under control and more die from it than among the non-deficient animals. Perhaps chickens, which have a higher biotin content in the liver than ducks, can increase their blood biotin level more rapidly and effectively than ducks, accounting for their greater resistance. *P. cathemerium* must be assumed to require a higher but narrower optimal range of blood biotin concentration than *P. lophurae*, since this parasite multiplies very rapidly at first in normal ducks. If this infection also increases the blood biotin level, then the abrupt decline in normal ducks can be explained partly on the basis of too high a concentration of biotin. In the biotin-deficient ducks, *P. cathemerium* at first multiplies more slowly than in normal ducks, presumably because the blood biotin level is below the optimal range. But as the biotin concentration increases in response to the infection, it enters the optimal range and the parasites multiply more rapidly. The deficient ducks then have difficulty in raising the biotin concentration above the optimal range and most of them die with a high parasite number in the blood. It is obvious that while

this simple hypothesis could explain the facts thus far available, much more information is needed before it can be taken very seriously

It is possible that biotin deficiency exerts some specific effects on cell systems, such as the lymphoid-macrophage system, which are intimately concerned with defense against malaria parasites (35), or that it interferes with protein metabolism and in this way with antibody formation (36) Either of these suppositions would encounter difficulties in explaining the initial slower multiplication of *P cathemerium* in the deficient animals Moreover, the evidence at present available indicates that the biotin level of the host affects the natural rather than the acquired resistance Attempts to produce relapse, by means of biotin deficiency, in animals recovered from infection with *P lophurae* failed Such experiments should, however, be repeated with a species of parasite (as *P gallinaceum*) which has a fairly high relapse rate

Any attempt to explain the effect of biotin on susceptibility to malaria must also recognize the fact that there are undoubtedly many other substances within the host the concentration of which also affects the susceptibility Some of these substances may well have a more striking effect than biotin, and the effect of the sum total of all such substances must determine the degree of susceptibility of a host to a parasite

The relation of nutrition to resistance to disease is still far from clear There can be no doubt that resistance to disease is greatly decreased in the presence of extensive nutritional deficiency, but under such conditions the complicating factors (such as inanition) are numerous and it is impossible to conclude that any specific relationship exists between a particular dietary factor and the degree of resistance to a particular infectious agent However, when a mild degree of a specific nutritional deficiency, unaccompanied by any general severe weakening of the animal, is accompanied by changed susceptibility to an infectious disease, one may be justified in assuming that a specialized kind of relationship exists between the level in the host of the nutritional factor concerned and the susceptibility of the host to invasion by the parasite Further justification for such an assumption is provided if, as in the present work, even extreme deficiency of some other nutritional factor does not lead to changed susceptibility There are in the literature relatively few examples of such specific relationships between the level in the host of a vitamin and the degree of susceptibility to an infectious agent Although Ackert, McIlvaine, and Crawford (37) in their original work on the effect of vitamin A deficiency in increasing susceptibility to helminth infections, failed to detect any effects of a subclinical degree of vitamin A deficiency, McCoy (38) found that rats depleted of vitamin A showed lowered resistance to *Trichinella spiralis* before there were any other signs of avitaminosis Boynton and Bradford (39) had similarly found that the lowered resistance of rats on a vitamin A deficient diet to infection with a bacillus of the *Mucosus capsulatus* group was apparent before any

other signs of vitamin deficiency The resistance of rats to infection with murine typhus rickettsiae was greatly lowered even by a mild degree of riboflavin deficiency, but was not affected by extreme vitamin A deficiency (40) Rats deficient in thiamin were more susceptible to rat leprosy (41) and mice partially deficient in thiamin or riboflavin were more susceptible to pneumococcal infection than animals receiving adequate amounts of these vitamins (42)

Of a somewhat different type are the results of Becker and his associates (43–45) on the effects of dietary factors on the extent of multiplication of the coccidial parasite of rats, *Eimeria nieschulzi* If rats were kept on a diet low in thiamin and pyridoxine, the addition of thiamin decreased the severity of infection and the further addition of pyridoxine decreased it still more However, if only pyridoxine was added, the infection was more severe than on the basal diet. In rats fed a diet partially deficient in pantothenic acid, the infection was less severe than in rats fed the same diet supplemented with calcium pantothenate. It has similarly been found that the mortality rate from poliomyelitis is higher in normal rats than in rats on a riboflavin-deficient diet (46), and that it is higher in rats on a high thiamin diet than in rats on a low thiamin diet (47) All these results, together with those reported in this paper, would certainly support the idea that the vitamins are among the substances whose concentration in a host animal influences the extent of growth or multiplication of certain parasitic agents This influence could be just as direct as the influence on a free living protozoon of the concentration of growth substances in its environment

Since individual animals are known to differ in their body level of growth factors, even though they are not deficient and are within the normal range, it may be that such differences help to account for individual differences in susceptibility

SUMMARY

Biotin-deficient chickens and ducks developed much more severe infections with *Plasmodium lophurae* than did non-deficient control animals While a very mild degree of biotin deficiency sufficed to increase susceptibility, even an extreme degree of pantothenic acid deficiency had no effect. Biotin deficiency also increased the susceptibility of ducks to *P cathemerium* In animals infected with *P lophurae*, the concentration of biotin in the plasma as well as in the red cells rose during the course of the infection, reached a peak at about the same time as the parasite number reached its peak, and then returned to normal as the infection subsided While the administration of additional biotin to animals partially deficient in biotin could be considered a specific measure tending to lessen the severity of infection with *P lophurae*, the injection of biotin into animals fed a diet adequate in this vitamin had no antimalarial effects, perhaps because the excess biotin was rapidly removed from the blood

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EXPLANATION OF PLATES

The photographs were made by Mr J A Carlile

PLATE 22

FIG 1 Left Chick fed casein diet 2*a* Right Chick fed egg white diet 2 Both 29 days old

FIG 2 Left Duck fed casein diet 4*a* until 11 days old, thereafter casein diet 4*b* Right Duck fed egg white diet 4 Both 20 days old

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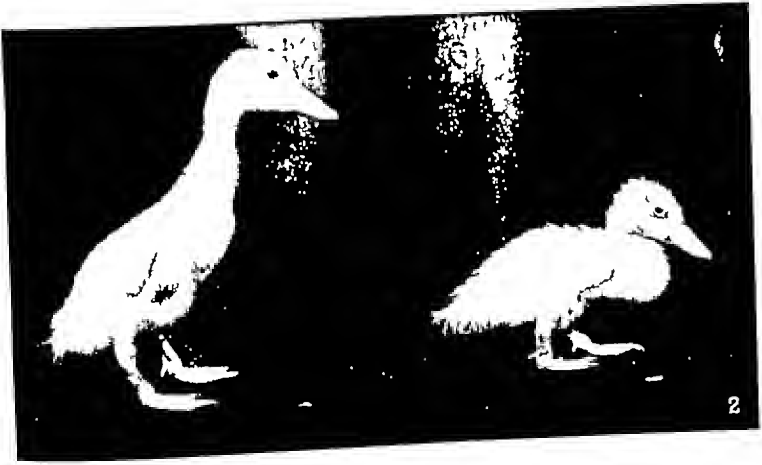


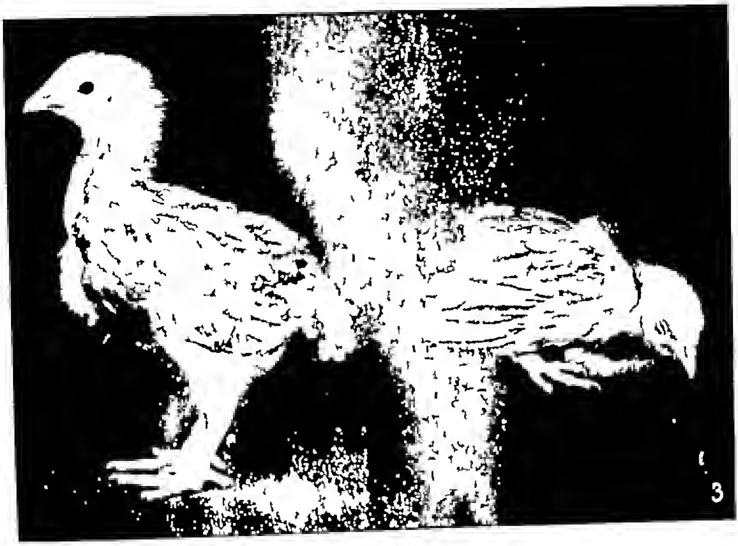
2

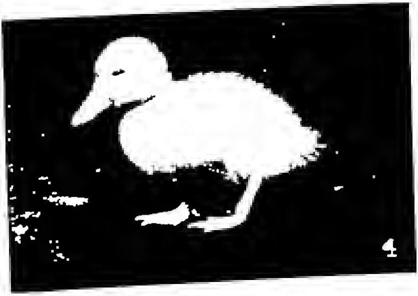
(Trager Influence of biotin upon susceptibility to malaria)

PLATE 23

FIG. 3. Left: Chick fed diet 5 I adequate in pantothenic acid. Right: Chick fed diet 5 deficient in pantothenic acid. Both 33 days old.

FIG. 4. Duck fed diet 5 deficient in pantothenic acid. 13 days old.





(Trager: Influence of biotin upon susceptibility to malaria)

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